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FOKI VITAMIN D RECEPTOR GENE POLYMORPHISMS AND METABOLIC HEALTH IN PREGNANT SAUDI WOMEN

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FOKI VITAMIN D RECEPTOR GENE POLYMORPHISMS AND METABOLIC HEALTH IN PREGNANT SAUDI WOMEN

A Dissertation Presented

by

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DEDICATION

To Baba and Mama: You kept my head held high throughout my life. It’s time to pay you back and make you proud.

To Layan, Faris, and Ghiath: Never give up on chasing your dream, no matter how hard it might get. Embrace it till you ace it.

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ABSTRACT

FOKI VITAMIN D RECEPTOR GENE POLYMORPHISMS AND METABOLIC HEALTH IN PREGNANT SAUDI WOMEN

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Lifestyles have been dynamically changing in the past few years in Saudi Arabia, and the prevalence of non-communicable diseases (NCDs), such as obesity, diabetes, hypertension, high cholesterol, and other cardiovascular diseases (CVD), is notably increasing. In fact, NCDs are affecting a growing number of people in SA, especially women of childbearing age. Vitamin D deficiency has also become pandemic and a major public health concern among the Saudi population despite the abundance of sunlight. The most vulnerable groups are pregnant women and their newborns. Previous studies have correlated low vitamin D status with a higher risk of adverse short- and long-term health consequences for both mother and child. Therefore, identification of novel genetic markers in women at risk of adverse pregnancy outcomes could lead to earlier identification of at-risk pregnancies and inform possible interventions to reduce adverse health outcomes.

The fact that vitamin D receptors (VDR) are present in almost every tissue of the human body has led to a debate about the extra-skeletal functions of vitamin D. To date, numerous studies have associated VDR gene polymorphisms to serious chronic illnesses. One candidate gene that has attracted interest in the literature is *FokI* VDR polymorphisms. *FokI* VDR, is capable of altering the protein structure and produces two structurally different variants: a longer (*f* allele) and a shorter (*F* allele), which is more effective and has higher transcriptional activity than the long variant (*f* allele).

Since there is scarce research regarding the potential association between *FokI* VDR polymorphism and adverse metabolic health outcomes in pregnant Saudi women, we hypothesized that Saudi mothers with poorer vitamin D status plus expression of the longer, less effective *f* version of the VDR protein would be at greatest risk of any adverse effects, such as vitamin D deficiency, gestational diabetes mellitus (GDM), and metabolic syndrome (MS).

We carried out a cross-sectional study in pregnant Saudi women and compared the association between the *FokI* VDR genotype and different metabolic health outcomes. The participants were randomly selected from primary health care centers and governmental hospitals scattered around the city of Riyadh during their first and second trimesters check-up between December 2013 and January 2016. The *FokI* VDR genotype was assessed, and its association with different variables, including vitamin D status, GDM, and MS and its components, were also analyzed.

In the first study, we investigated the association between *FokI* VDR gene polymorphism and serum 25-hydroxyvitamin D (25(OH)D) levels in 345 pregnant Saudi women (273 with vitamin D deficiency and 72 non-deficient subjects) in their first

trimester. About 79% of the women studied were vitamin D deficient (25(OH)D < 50 nmol/L). Our findings indicated that the *Ff* genotype and the combined variant genotype (F*f*+ *ff*) showed a significant decrease in the risk of developing vitamin D deficiency (*FF* vs. *Ff*, OR = 0.44, 95% CI = 0.20–0.94, *P* = 0.035, *FF* vs. *Ff*+*ff*, OR = 0.42, 95% CI = 0.20–0.88, *P* = 0.022), after adjusting for confounding factors that had significant effect on altering vitamin D status.

In the second study, we examined whether there was a difference in the risk of having GDM among the various *FokI* VDR genotypes in 108 GDM patients and 260 healthy pregnant women in their second trimester. We found no significant difference in risk. However, within the group of patients carrying the minor *ff* genotype, we found that serum 25(OH)D was significantly and inversely associated with fasting serum glucose and hemoglobin A_{1C}. Vitamin D deficiency was highly prevalent among study participants amounting to 85.3%. It was also significantly associated with GDM (*P*=0.016) and glucose indices such as fasting insulin, HOMA_IR and HOMA_β.

In the third study, we investigated the association of the *FokI* VDR genotype as a risk factor for MS and its components in 368 pegnant Saudi women (44 with MS and 324 without MS) in their second trimester. We found that the minor *ff* genotype was a significant risk factor for MS (*P* = 0.039; OR = 2.91; 95% CI, 1.05–8.1).

Together, the results of the previous studies suggest that *FokI* VDR gene polymorphism could be a risk factor for vitamin D deficiency, poorer glucose homeostasis, and MS in the Saudi population.

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CHAPTER 1

CHALLENGES FACING PREGNANT SAUDI WOMEN

Saudi Arabia is a high-income country with a well-developed health care system, including free antenatal care for all pregnant Saudi women at primary healthcare centers as well as referral to government hospitals.[1] Moreover, in the past few years there has been a significant improvement in the educational and employment status of the Saudi female population.[2] These enhancements in medical care and sociodemographic characteristics have clearly manifested in overall pregnancy outcomes, including the maternal mortality ratio, which declined from 23/100,000 in 2000 to less than 13/100,000 in 2015.[2] Despite this, epidemiological studies conducted in Saudi Arabia found a high stillbirth rate of 12/1000 live births, which is high when compared with similar high-income countries, with the reason attributed to a high prevalence of maternal diabetes and obesity.[1]

The rapid urbanization and advancement in socioeconomic status has resulted in a significant change in Saudi lifestyle behaviors, including increased consumption of high-energy dense foods, physical inactivity, and the subsequent emergence of non-communicable diseases (NCDs) such as obesity, diabetes, hypertension, high cholesterol, and other cardiovascular diseases (CVD).[1] NCDs have thus become a major public health problem and are now the primary cause of death among adults in Saudi Arabia.[3] In fact, the death toll from NCDs reached 78% during 2014, and CVDs were responsible for about (46%) of these deaths.[4] The most alarming fact is that these risk factors are

prevalent at younger ages,[5] and it is well known that Saudi Arabia has a young population. More than half of the population is younger than 25 years,[6,7] 78.3% of women are under the age of 40,[8] and the over-65 group constitutes only 3.7% of the total population.[1] These health challenges are concerning in this specific group of women. Therefore, prevention and early detection should be prioritized to improve health in the Kingdom of Saudi Arabia (KSA).

In addition to the previously mentioned health problems, vitamin D deficiency is highly prevalent in KSA, particularly in females and in the younger age groups.[9] Al-Faris conducted a cross-sectional study of 160 pregnant Saudi women and found that younger pregnant women (aged 20–34 years) had a significantly higher prevalence of vitamin D deficiency compared with older women (aged 35–49 years) ($P = 0.029$).[10] The author attributed this observation to unhealthy dietary patterns among younger women, characterized by high fast food consumption and less vitamin D intake or supplementation.[10]

The fact that the previously mentioned health problems are more common at younger ages deserves immediate attention and investigation of the underlying mechanisms. Specifically, according to Al Quaiz et al., Saudi women are at a greater risk of developing CVD and diabetes compared to a decade ago**,** with a notable increase in obesity compared to men.[6] Moreover, a report published by the World Health Organization in 2015 revealed that life expectancy among Saudi Arabian women is still lower than that observed in many developed countries (Japan = 86.8, Australia = 84.8, United Kingdom = 83, United States of America = 81.6, United Arab Emirates = 78.6, KSA = 76 years).[11] This chapter will address the health problems prevalent among

pregnant Saudi women and the risk factors that contribute to adverse health outcomes for mothers and their newborns.

1.1 Vitamin D Deficiency in Pregnancy

1.1.1 Prevalence and Diagnosis

Vitamin D deficiency is considered a public health problem around the world.[12] Vitamin D status has been investigated in numerous countries based on the concentration of serum 25-hydroxyvitamin D (25(OH)D).[13] There is a debate in the literature regarding the definition of vitamin D deficiency. Most researchers agree that a 25(OH)D concentration < 50 nmol/L is an indication of vitamin D deficiency, whereas a 25(OH)D concentration of 51–74 nmol/L is considered to indicate insufficiency; concentrations > 75nmol/L are considered to be sufficient.[13] This is based on the observation that intestinal calcium absorption is maximized above 80 nmol/L, and parathyroid hormone (PTH) concentrations in adults continue to decline and reach their nadir at ≈75–100 nmol/L.[13]

As there are no tailored recommendations for the diagnosis and treatment of vitamin D deficiency in the Middle Eastern region, the Prince Mutaib Chair for Biomarkers of Osteoporosis (PMCO) in King Saud University (KSU), Riyadh, KSA, together with local experts and in cooperation with the European Society for Clinical and Economic Aspects of Osteoporosis and Osteoarthritis (ESCEO), organized a panel to resolve this issue and established a reference range for the cut-off values of serum 25(OH)D based on the following: Vitamin D sufficiency is defined as circulating serum 25(OH)D ≥ 50 nmol for the general population and vitamin D adequacy as serum 25(OH)D > 75 nmol/L for the frail and osteoporotic elderly. The panel also recommended vitamin D supplementation of 1000–2000 IU/day (vitamin D2 or vitamin

D3) for pregnant Saudi women, which it claimed would be feasible, safe, and could improve maternal and fetal outcomes.[14]

In KSA, two meta-analyses were conducted to assess the prevalence of vitamin D deficiency across all demographics of the Saudi population.[15,16] Both systematic reviews used the same cut-off values for determining vitamin D status and were conducted around the same years. Strangely, however, they reported contentious results. In the first meta-analysis, which included 16 articles and was carried out between 2008 and 2015,[15] Al-Alyani et al. found that the overall rate of vitamin D deficiency in the healthy Saudi Arabian population was 63.5%.[15] Meanwhile, the other meta-analysis, which was conducted by Al-Daghri between 2011 and 2016 and included a review of 13 articles, showed the overall prevalence of vitamin D deficiency to be 81.0%.[16] The discrepancy between these numbers is relatively large and could be due to a non-unified cut-off for vitamin D deficiency, different diagnostic methodologies, and varying study designs and qualities. [17]

Although this issue seems controversial, no one can deny that KSA, the land of sunshine, has alarming rates of vitamin D deficiency in most of its population, across all ages and in both genders. Specifically, Saudi females have higher rates of severe vitamin D deficiency (< 25 nmol/L) when compared to males (44.6% vs. 28.3%, $p < 0.000$), as reported in a retrospective observational study of 10,709 patients.[18] This issue needs to be highlighted and carefully addressed, especially in vulnerable groups that appear to be more susceptible to developing vitamin D deficiency, such as women of childbearing age, pregnant women, and pregnant women at risk of GDM.[19] Previous studies have shown

that vitamin D deficiency occurs in up to 90% of pregnant women and depends on many factors, such as the country of origin and lifestyle.[20]

In KSA, vitamin D deficiency affects approximately 78% of Saudi females among various age groups.[21,22] Studies of women of childbearing age found a variable prevalence of vitamin D deficiency, from one-third[22] to three-fourths[23] of the population studied. Al-Faris found that over 90% of pregnant Saudi women were either vitamin D deficient (serum 25(OH)D level < 50 nmol/L) or insufficient (serum 25(OH)D level = 50–74 nmol/L).[10] In another study, which was conducted on a larger sample of 1,097 pregnant Saudi women living in Riyadh, the capital city of KSA, Fouda et al. found that almost 85% of pregnant subjects and 88% of their neonates were vitamin D deficient (serum 25(OH)D level < 50 nmol/L).[17] Similarly, Al-Wassia and Abo-Ouf and Alsheikh et al. reported a very high prevalence of maternal vitamin D deficiency ranging, from 86.4% to 90.5% in pregnant Saudi women. [24,25] Al-Wassia and Abo-Ouf also found that 86% of infants were vitamin D deficient.[24] Although numerous studies have assessed the prevalence of vitamin D deficiency in KSA, information is still lacking regarding vitamin D status throughout pregnancy, as most previous studies have measured vitamin D once in the third trimester or at delivery.

1.1.2 Causes

Maternal vitamin D status varies across different pregnancy trimesters, and the risk of vitamin D deficiency increases due to the high maternal and fetal demands.[25] Aside from this, vitamin D levels are usually affected by several factors. The major risk factors associated with vitamin D deficiency in KSA have been agreed upon by several investigators and are related to Saudi customs.[10,16,17] KSA receives abundant sunshine

year round, and in summer months the temperatures often rise above 50°C. Therefore, Saudis avoid sun exposure and limit their outdoor activities during the daytime.[10] In addition, due to cultural and religious reasons, the majority of Saudi women wear dark veils that cover nearly all their body parts, which block sunlight and necessitate increased circulating 25(OH)D production from sun exposure.[16,23,26] Certainly, there are many other risk factors that affect vitamin D status in Saudi women, including dietary habits, dark skin pigmentation, pregnancy, physical inactivity, obesity, and lack of government regulation for vitamin D fortification of food.[18, 25]

Interestingly, Hussain *et al.* found that vitamin D deficiency was more common and severe in Saudis than in non-Saudis living in the same city of Riyadh.[18] This observation sheds light on the influence of genetic variantiations, such as vitamin D receptor gene polymorphisms, which might play a role in vitamin D metabolism and disease susceptibility.[25] Sadat-Ali *et al.* were the first to investigate the genetic influence on vitamin D status in the Saudi population.[27] They found that several single nucleotide polymorphisms (SNPs) in vitamin D-related genes were significantly associated with vitamin D deficiency compared to the population with normal vitamin D levels.[27] However, there have been no studies to confirm such a relationship in KSA with larger sample sizes and controlling for different confounders that might mask vitamin D status, such as the risk factors mentioned earlier in this section.

1.1.3 Adverse Perinatal Outcomes

Vitamin D deficiency during pregnancy has been associated with several maternal and offspring complications that persist later in life.[28] Stores of vitamin D in newborns are dependent on maternal vitamin D status.[20] Maternal serum and cord blood 25(OH)D

are highly correlated, and both maternal serum and cord blood 25(OH)D levels increase after maternal vitamin D supplementation.[29] Indeed, two recent cross-sectional studies found a significant association between maternal serum 25(OH)D and neonatal cord blood Vitamin D levels in pregnant Saudi women.[17,24]

It has been also suggested that low serum 25(OH)D in the mother might cause reduced transfer of 25(OH)D to the fetus, leading to impaired growth, low infant birth weight, delayed bone ossification and congenital rickets, abnormal tooth enamel formation, and lower bone mineral content.[30] Case reports show that neonatal complications from extreme maternal vitamin D deficiency can be life threatening, e.g., severe hypocalcemic fits with high risks of resultant brain damage and neonatal heart failure.[29]

Aside from causing adverse skeletal effects, vitamin D deficiency during pregnancy has also been linked with a number of maternal problems, including infertility, preeclampsia, gestational diabetes, caesarean section, bacterial vaginosis, postpartum depression, neonatal hypoglycemia, low birth weight, and preterm birth.[10,31-33] Fortunately, a recent study found that supplementing pregnant women with vitamin D and increasing maternal serum 25(OH)D levels to ≥ 100 nmol/L was associated with a 60% reduction in preterm birth risk in a large, diverse population of women.[34]

In KSA, Alsheikh et al. studied the effect of hypovitaminosis D on adverse pregnancy and neonatal outcomes and found that hypertensive pregnancy disorders and a history of miscarriage were significantly associated with vitamin D deficiency and insufficiency when compared to women with normal vitamin D levels.[25]

1.2 Gestational Diabetes Mellitus

1.2.1 Prevalence and Diagnosis

The American Diabetes Association (ADA) defines GDM as "carbohydrate intolerance resulting in high blood sugar (hyperglycemia) of variable severity, with onset or first recognition during pregnancy."[35] It is usually diagnosed between 24 and 28 weeks of gestation.[35] GDM is recognized in the global population as ranging from 1% to 14% of all pregnancies, depending on the ethnicity and the GDM diagnostic tests used.[36] Hence, GDM is considered to be one of the most common complications of pregnancy.[37] When compared to adverse maternal outcomes, GDM is the most prevalent complication seen in pregnant Saudi women (11.1%).[25] In fact, in their large multicenter cohort study, Wahabi et al. reported that the prevalence of GDM (24%) and pre-GDM (4.3%) in pregnant Saudi women is among the highest in the world and is associated with high maternal and neonatal morbidities and mortalities.[1] Another study conducted in KSA showed that 18.7% of all pregnancies led to a GDM status.[38] This alarmingly high rate exceeds the prevalence that is reported in the United States and Europe.[39] In fact, the prevalence of GDM in the United States is far less than in KSA, ranging from 3.47% to 7.15%.[40]

Although GDM affects only a sub-group of the population, its prevalence is rising along with soaring rates of obesity and Type 2 diabetes mellitus (T2DM).[41] For example, In the United States, Getahun et al. reported that the prevalence of GDM increased gradually between 1989–1990 and 2003–2004 by 122%, from 1.9–4.2%, respectively.[42] Considering the high burden of T2DM in the Saudi population and the fact that the occurrence of GDM is directly related to that of T2DM, a high rate of GDM is

anticipated to continue to increase in pregnant Saudi women.[1] The prevalence of GDM is expected to increase fourfold in subsequent decades,[43] stressing the importance of further research into its pathophysiology.

1.2.2 Causes

The most commonly reported risk factors for the development of GDM are high maternal age (> 35 years), obesity, ethnicity, parity, family history of type 2 diabetes and/or GDM, previous delivery of a macrosomic infant, and diagnosis of polycystic ovarian syndrome (PCOS).[44] However, these well-known risk factors are only responsible for about 50% to 60% of the risk of future GDM.[45] Other factors mentioned in observational studies that are independently associated with higher GDM risk include the following: higher triglycerides,[46] inflammation,[47] gestational weight gain (GWG),[48] and low vitamin D levels.[19] Studies from KSA reported that the most commonly observed risk factors were increased maternal age, higher body weight and BMI, previous history of GDM, macrosomia, and multiparae.[49,50] The question remains: Why are certain ethnic groups, such as pregnant Saudi women, at a higher risk of GDM?

The underlying pathophysiological mechanisms for GDM are poorly understood and seem to be multi-factorial.[51,52] Nevertheless, it is well known that gestational steroid hormones coupled with placental outcomes (hormones and cytokines) are altered by insulin secretion and resistance.[53] When insulin secretion is unable to meet increased maternal needs, secondary to increased resistance, GDM occurs.[51,53] As a result, an increased request for insulin during pregnancy places an additional burden on pancreatic β-cells, which can lead to pancreatic dysfunction.[51] Previous data have linked vitamin D deficiency to GDM, but some studies have not found this association. A recent systematic

review found that vitamin D deficiency (serum 25(OH)D <50 nmol/L) in pregnancy is associated with a 1.61-times increased risk of GDM.[54] However, this association remains inconclusive and requires more studies to elucidate the impact of low vitamin D status on the occurrence of GDM.

1.2.3 Adverse Perinatal Outcomes

Gestational diabetes (GDM) complicates 3–14% of pregnancies, and it is responsible for an important proportion of fetal and maternal morbidity and mortality in both the short and long term.[42] Short-term effects include maternal GDM complications, such as pregnancy-induced hypertension, preeclampsia, urinary tract infections, and cesarean deliveries;[55] long-term complications include a 35–80% higher risk of recurrent GDM,[56] a 70% higher risk of developing T2DM within 10 years after delivery,[53] and a higher risk of developing metabolic syndrome.[57] In the offspring of GDM mothers, there can also be short-term complications, such as fetal macrosomia, birth trauma, shoulder dystocia respiratory distress syndrome, congenital malformation, and neonatal jaundice as well as an increased risk of neonatal intensive care unit (NICU) admission.[42] Meanwhile, long-term complications for the child can include childhood obesity, metabolic syndrome, T2DM, and CVD.[58]

According to a study performed in KSA, GDM was a major contributor to perinatal death, congenital malformation, and neonatal jaundice.[59] Similarly, most studies conducted in KSA reported that GDM affected both the mother and fetus. GDM mothers were more likely to have cesarean deliveries, and the infants born to such mothers were at higher risk of developing hypoglycemia, higher birth weight, hyperbilirubinemia,

macrosomia, and higher NICU admissions when compared to infants born to non-GDM mothers.[49]

Interestingly, not only mothers with GDM suffered from perinatal complications; pregnant Saudi women with pre-GDM also experienced hypertensive disorders during pregnancy, induction of labor, cesarean section delivery, preterm delivery between 34 and 36 weeks, stillbirth rate, neonatal admission to NICU, macrosomia, low APGAR scores, and shoulder dystocia when compared to nondiabetic women.[1]

1.3 Metabolic Syndrome

1.3.1 Prevalence and Diagnosis

Metabolic syndrome (MS) is a cluster of risk factors for T2DM and CVD.[25,60] The National Institutes of Health (NIH) defines MS as having at least three of the following risk factors: central obesity, elevated triglycerides, low high-density lipoprotein cholesterol, hypertension, and elevated fasting plasma glucose.[61]

MS is considered an epidemic of the 21st century and a huge burden that needs to be carefully addressed in both developed and developing countries.[60] According to the International Diabetes Federation (IDF), a quarter of the adult population worldwide suffers from MS.[62] The prevalence estimates of MS differ depending on which defining criteria are utilized. Using the IDF's definition of MS, according to the National Health and Nutrition Examination Survey (NHANES), the prevalence of MS rose significantly from 25.3% between 1988 and 1994 to 34.2% between 2007 and 2012 among US adults.[63]

In KSA, the prevalence of MS is much higher than in the US, with rates varying

between 25.5% and 55% when using the same IDF definition.[64] After reviewing different studies and using various defining criteria, Bahijri and Raddadi found that the overall prevalence of MS in the Saudi population varies from 13.6% to 57%.[65] The authors attributed the difference in prevalence to variations in the targeted populations, geographical location, gender, age groups, and the criteria used to define MS.[62] Irrespective of the defining criteria of MS, the calculated prevalence is very high among the Saudi population, particularly among Saudi females.[62] This issue raises concern for women of childbearing age and pregnant women in KSA, as the detection and screening of MS during pregnancy has not been addressed in the literature. Studies on pregnancy from around the world have shown that women with MS have a higher risk of having newborns with perinatal adverse outcomes, demanding attention and special care.[60] Therefore, diagnosis during pregnancy is essential in terms of determining the women at risk of cardiovascular and metabolic changes.[66]

1.3.2 Causes

Despite the fact that the pathogenesis of MS has not been studied thoroughly,[67] it is known to be a multi-factorial disorder influenced by the synergistic effects of genetic and environmental risk factors.[62,65]

Environmental components such as changes in diet and lifestyle explain the increased prevalence of MS in Saudi Arabia. [62,65] As mentioned earlier, the prevalence of MS is increasing worldwide and is associated with the rising incidence of obesity epidemic along with population aging and urban housing.[57,60] Surprisingly, the overall prevalence of obesity in adult females in KSA has been previously reported as one of the highest worldwide.[4,6,25] Moreover, obesity rates are higher in Saudi females than in their

males counterparts.[5,68] A large national survey conducted in KSA revealed that 33.5% of women were obese and 28.7% were overweight.[8] These high rates of obesity in this population are extremely shocking and must be addressed, especially for pregnant Saudi women.

Over all, it is estimated that maternal obesity complicates 33% of all pregnancies, having detrimental effects on the metabolic health of the mother and offspring.[61] Maternal obesity has been associated with macrosomia, delivery by cesarean section, pregnancy-induced hypertension, and higher rates of early miscarriage and congenital anomalies, including neural tube defects, which can lead to fetal death.[50,60,69]

Most studies have agreed that the high prevalence of obesity among Saudi women could be attributed to unhealthy dietary patterns and physical inactivity.[3,8,62] Al-Quaiz reported a very high rate of physical inactivity, reaching upwards of 98.1% in Saudi females.[70]

The genetic components of MS have been investigated in the Saudi population with relatively very small study sizes. We will elaborate more on this topic in Chapter 2.

1.3.3 Adverse Perinatal Outcomes

It is vital to identify women with MS, as they are at twice the risk of developing CVD over the next 5–10 years.[57] According to World Health Organization (WHO), CVDs are the primary cause of death in women worldwide, accounting for 33.2% of female deaths in 2008.[71] In KSA, the estimated prevalence of CVD is around 42%, with a high proportion of young women aged 30 years and above affected.[72] Strangely, the mean age of CVD incidence was estimated to be ten years younger in the Gulf region when

compared to that in European countries.[72] Women with MS also have a higher risk of developing GDM,[69] and thus they have a 30% risk of developing future T2DM.[57,60]

There are few studies on metabolic syndrome in pregnancy. Dane et al. found that pregnant women with MS had significantly higher rates of GDM and preeclampsia when compared with women with normal pregnancies.[66] Moreover, Tavares et al. reported finding adverse perinatal outcomes in 14.1% of pregnancies with MS.[60]

Overall, women in KSA face serious health challenges, such as vitamin D deficiency, GDM, and MS. These conditions are alarmingly widespread, especially at young ages. To develop strategies for primary prevention, more research is needed on younger women, and special attention should be focused on pregnancy to prevent the burden of disease in coming years.

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CHAPTER 2

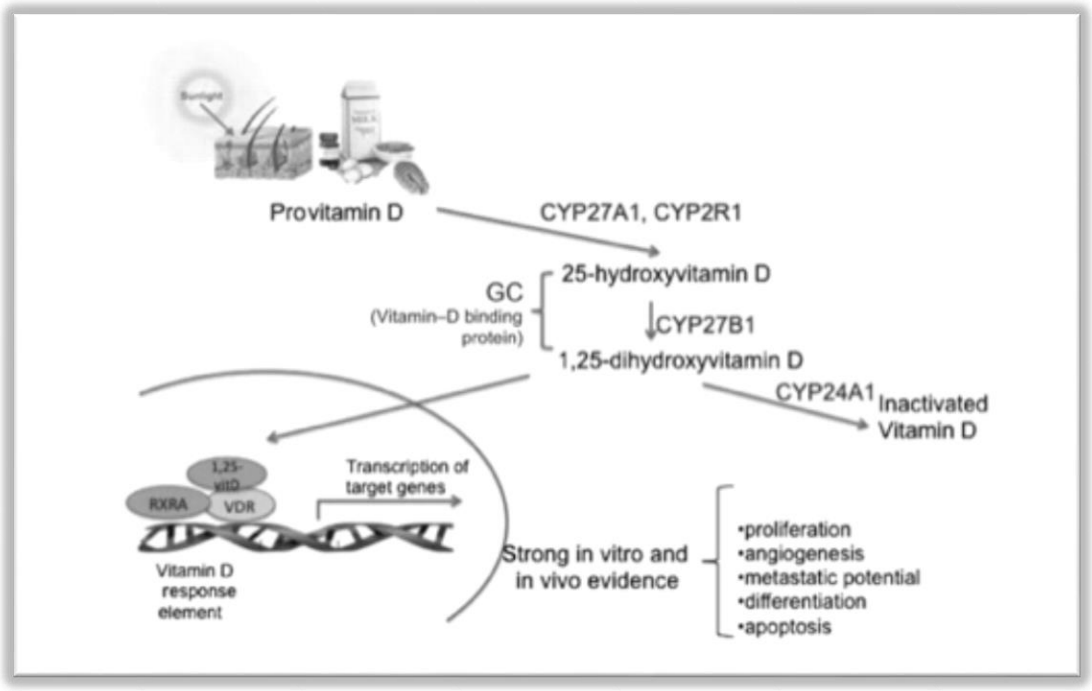
FOKI VITAMIN D RECEPTOR GENE POLYMORPHISMS AND ITS ASSOCIATION WITH NON-SKELETAL OUTCOMES

2.1 Vitamin D Metabolism Pathway

It has been long known that the effect of genetic variation in the vitamin D synthesis and metabolism pathway on circulating concentrations is not clear.[1] The two forms of vitamin D, which is either synthesized in the body from adequate exposure of the skin to sunlight (vitamin D_3; cholecalciferol) or obtained from diet (vitamin D_2; ergocalciferol), are both prohormones that are biologically inactive.[2] Both vitamin D_3 and D_2 are hydroxylated twice to obtain their biologically active form, calcitriol, or what is known as 1,25-hydroxyvitamin D ($1,25(OH)_2D$).[3] The first hydroxylation occurs in the liver, where the enzyme 25-hydroxylase (CYP2R1) hydroxylates vitamin D to 25-hydroxyvitamin D (25(OH)D; calcidiol), which is the circulating form of vitamin D and the most reliable indicator reflecting vitamin D status.[4] The second hydroxylation occurs primarily in the kidneys or at the local tissue level and is performed by the enzyme 25(OH)D-1-α-hydroxylase (CYP27B1) to yield $1,25(OH)_2D$.[4] The active hormone, $1,25(OH)_2D$, is then released in the blood, where it binds to the vitamin D–binding protein (DBP) and arrives at the targeted cells to apply its endocrine functions through the vitamin D receptor (VDR; **Figure 2.1**).[5,6] This process is vital in regulating many genes that play significant roles in controlling cellular growth and differentiation.[5,6] Thus, any genetic variation or mutation in this metabolic pathway has the capability of altering serum 25(OH)D concentrations.[1]

Figure 2.1: Vitamin D metabolism and genes involved in vitamin D metabolic pathways

(Adapted from Hossein-Nezhad & Holick, 2013)[6]



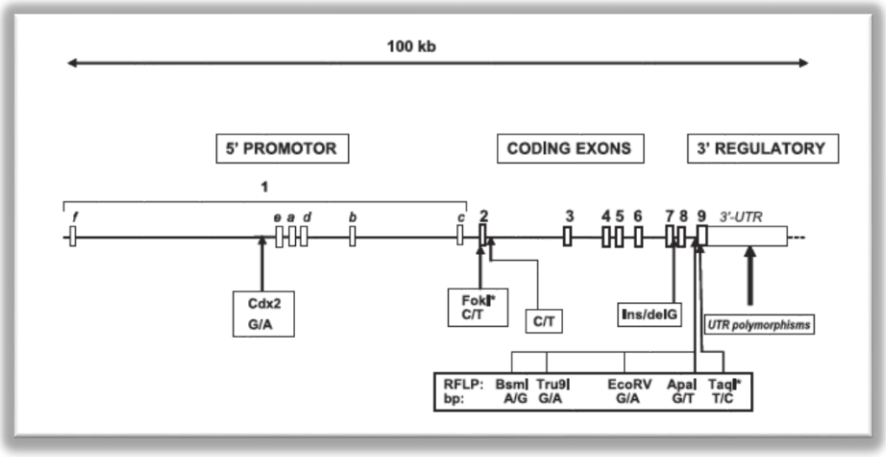
2.2 Vitamin D Receptor (VDR) Gene Polymorphisms

VDR, a member of the steroid/thyroid hormone nuclear receptor family, is ubiquitously expressed in nearly all nucleated human cells at fluctuating concentrations.[7] The VDR gene is considered to be a candidate for controlling responses to vitamin D.[8] As mentioned in the previous section, VDR binds to the active form of $1,25(OH)_2D$ and, in turn, the ligand-activated VDR forms a heterodimer with the retinoid X receptor.[9] The heterodimer binds to vitamin D–response elements in the promoter regions of vitamin D–dependent target genes and regulates the expression of various genes related to vitamin D metabolism.[9,10] VDR is also known to be involved in bone and calcium homeostasis, cell differentiation, immunomodulation, and the control of other hormonal systems.[11]

The VDR gene, which is at the chromosomal locus of 12q12-14, includes 2 promoter regions, 8 coding exons (namely, 2–9), and 6 untranslated exons (1A–1F).[12] Several single nucleotide polymorphisms (SNPs) have been identified in the VDR gene, including 3 SNPS located at the 3′ untranslated region (3′ UTR) of the gene, namely *Bsm*I (A > G, rs1544410), *Apa*I (A > C, rs7975232), and *Taq*I (T > C, rs731236), as well as another polymorphism known as *Fok*I (C > T, rs10735810), which is located within the 5′ end of the coding exons near the promoter region (**Figure 2.2**).[13]

Since VDR is found in nearly every human tissue, researchers investigated its functional significance and potential effects on disease susceptibility and the extraskeletal functions of vitamin D.[9] To date, numerous systematic reviews of the literature as well genome-wide association studies have linked VDR polymorphisms to various disease outcomes, such as bone mineral density, cardiovascular diseases, metabolic syndrome (MS), asthma, increased risk of T2D, an unfavorable lipid profile, hypertension, obesity, periodontitis, risk of multiple sclerosis, and malignancy.[14–16] In the next section, we will focus on *Fok*I polymorphism in VDR and its association with adverse health outcomes in pregnancy.

Figure 2.2: Structure of the VDR gene and position of single nucleotide polymorphisms (Adapted from Uitterlinden et al., 2004)[13]



2.2.1 *Fok*I VDR Gene Polymorphism

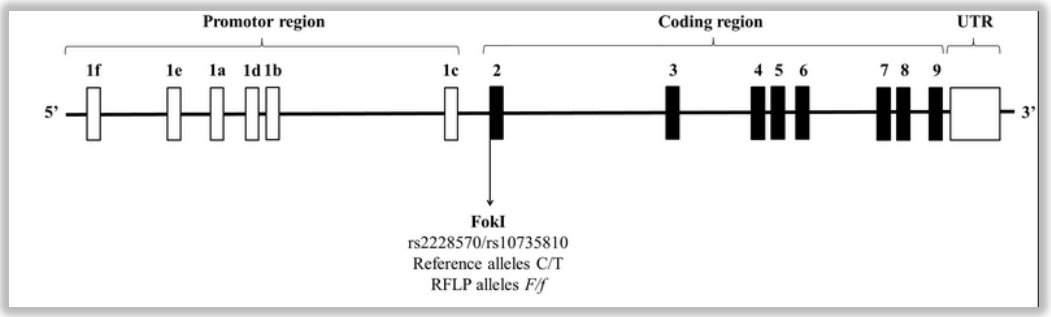
The *FokI* SNP (rs10735810 merged into rs2228570), which is defined by the restriction enzyme, is located in exon 2 of the coding region of the VDR gene (**Figure 2.3**).[17] It is uniquely different from the other SNPs for two reasons. First, it is the only polymorphism that is not linked to any of the other VDR variants.[18] Second, it is the only known VDR polymorphism, in contrast to other variants, that is translated into two different VDR protein products, thus affecting the VDR protein structure and function.[11]

In regards to this, VDR *Fok*I is considered one of the most important functional regions of the gene.[18] In fact, it is the only known VDR gene polymorphism that leads to the production of an altered protein.[17] In other words, it can generate two structurally distinct isoforms: a shorter *F* allele or a longer *f* allele protein.[19] In the *ff Fok*I genotype, translation initiation occurs at the first ATG site, giving rise to a full length VDR protein comprised of 427 amino acids.[17] On the contrary, in the *FF Fok*I genotype, translation begins at the second ATG site instead of the first, resulting in a shortened protein with 3 fewer amino acids (427 versus 424 amino acids).[17] The mutation in the *ff Fok*I VDR polymorphism results in a cytosine-to-thymine (C→T) nucleotide change in exon 2 of the

coding region of the VDR gene and creates a second upstream start site due to a codon change from ACG → ATG.[13] This start codon change causes the expression of a VDR protein that is 3 amino acids longer and has been shown to reduce transcriptional activity and to be less responsive to $1,25(OH)_2D$ than the shorter *F* allele version in activating target gene expression.[20] In fact, the *F* allele variant is 1.7-fold more active than the *f* allele.[15,21] However, various studies have shown inconsistent findings regarding the functional significance of the *Fok*I polymorphism,[9] although most data indicate that the *F* allele is more effective than the *f* allele in transactivation of the $1,25(OH)_2D$ signal.[13–19] Thus, women with the *F* allele may have better metabolic outcomes regarding bone health, glucose homeostasis, and inflammatory response.[9] Still, the number of publications that have examined the role of VDR polymorphisms in pregnancy is very limited. The aim of this chapter is to review the association of VDR *Fok*I polymorphism and metabolic disorders in the general population while shedding light on the limited research that has been done on pregnant women.

Figure 2.3: Location of the VDR gene *Fok*I polymorphism

(Adapted from Colombini et al., 2014)[17]



2.2.1.1 *Fok*I VDR Gene Polymorphisms and Vitamin D Deficiency

Twin- and family-based studies have previously found that heritable factors have a considerable effect on vitamin D status.[15,19] Depending on the VDR variant, it could either increase or decrease the serum 25(OH)D levels.[15,19]

The VDR *FokI* polymorphism, either individually or in combination with other VDR variants, has been investigated in a few studies and showed inconsistent results regarding their association with serum 25(OH)D levels or vitamin D deficiency. Recent reports from China,[1,22] Saudi Arabia,[23] the United States,[24] and the United Arab Emirates[25] have failed to detect any significant association between VDR *Fok*I polymorphism and circulating levels of 25(OH)D. To our knowledge, there is only one study that assessed the association between VDR *Fok*I polymorphism and vitamin D status in Iranian women during their gestation periods.[26] However, Aslani et al., who conducted this study, did not detect any significant differences in 25(OH)D levels between carriers of the *F* and *f* alleles among healthy pregnant Iranian women and GDM patients in their second trimester.[26]

In contrast, there is consistent evidence that the VDR gene *Fok*I polymorphism is significantly associated with 25(OH)D levels in adult patients with multiple sclerosis,[27–29] systemic lupus erythematosus,[30] and autism spectrum disorder in children.[18] The unified finding for all these studies indicated that the mutant *ff Fok*I genotype had significantly higher levels of serum 25(OH)D compared to *FF* and *Ff* carriers.[18,27–30] Some of these authors reported that the reason for higher levels of serum 25(OH)D in mutant *f* allele

carriers may be attributed to its compensatory response due to reduced VDR activity and distorted signaling pathways.[18,27]

On the other hand, few studies have reported a significant association between *ff* genotype carriers and low values of serum 25(OH)D when compared to the *FF* genotype in patients with T2DM,[8,31] patients with inflammatory bowel disease,[32,33] and patients with chronic kidney disease.[34] In fact, two of these studies demonstrated a significant association between vitamin D deficiency and the combined mutant genotype (*Ff* + *ff*) of *Fok*I in the Chinese patients with Crohn's disease[32] and ulcerative colitis.[33] Similarly, another study reported an increased risk of developing severe vitamin D deficiency (< 38 nmol/L) among chronic kidney disease patients carrying *Ff* and combined *Ff* + *ff* genotypes compared to healthy participants in the South African population.[34]

The inconclusive findings of all the studies could be attributed to their modest sample sizes; variability of the population and ethnic groups studied; and the failure to adjust for risk factors that alter serum 25(OH)D, such as vitamin D intake, use of supplements, latitude, seasonal variations, sun exposure, and others.[1] Additionally, most of the research to date has assessed the association between 25(OH)D and the VDR *Fok*I polymorphism on unhealthy subjects, and the lack of an association could be related to disease confounders.

Future prospective cohort studies on healthy subjects are required to clarify the primary genetic predictors of 25(OH)D concentrations, including the assessment of genes involved in vitamin D metabolism. It is vitally important to consider the proper

adjustments to be made for important confounders that are known to alter vitamin D status to appreciate the effects of genotype on vitamin D levels.

2.2.1.2 *Fok*I VDR Gene Polymorphisms and Gestational Diabetes

GDM is considered to be a serious public health problem associated with higher incidence rates of perinatal mortality and morbidity.[35] As previously mentioned, VDR is expressed in many human tissues, including the ones involved in the regulation of glucose metabolism, such as pancreatic β cells.[8,19] VDR is also known to alter glucose homeostasis via the insulin-like growth factor system.[8,12]

Since T2DM and GDM are very much alike in terms of sharing a similar pathophysiology, polymorphisms in genes that cause a predisposition for T2DM are closely related to GDM vulnerability.[12] Moreover, numerous studies were conducted to prove an association between VDR *Fok*I polymorphism and T2DM but with rather equivocal results.[36] Studies that have confirmed a significant association between VDR *Fok*I polymorphism and T2DM were carried out on Chinese[37] and Emarati[38] populations. In fact, the latter study found that the mutant *f* allele and *ff* genotype of VDR *Fok*I polymorphism were significantly associated with 85% increased risk of T2DM when compared to the control group ($P = 0.0007$),[38] whereas no significant association has been reported in Italian[39] or Indian populations.[40] According to a meta-analysis, the inconsistent evidence in the previous studies could be due to strong intra-individual variability of vitamin D status, which could probably affect the expression of VDR variants, as well as substantial inter-ethnic variance in the samples studied.[36]

Studies have shown a potential role of *FokI* polymorphism of the VDR gene in the pathogenesis of GDM, yet they remain scarce and inconclusive. Only three studies were available, and one was conducted in Saudi Arabia. The first study was conducted by Aslani and colleagues on 303 Iranian pregnant women (142 GDM patients and 161 healthy subjects) and found that the *ff* genotype was more common in GDM patients compared to the healthy subjects (OR = 1.783, 95% CI = 0.774–4.107). [26] Interestingly, healthy subjects had higher frequencies of the *F* allele (78.6% vs. 72.2%, [$P < 0.06$]), suggesting that the *F* allele may have a role in decreasing the incidence of GDM.[26] The authors attributed the reason for the association between the mutant *ff Fok*I genotype and a higher risk of GDM to the dysfunction of the *f* VDR variant. Because the mutant *f* allele is longer in structure and 40% less active than the shorter *F* allele, *f* allele carriers are therefore predisposed to a higher risk for GDM.[26] The second recent study, whose results were in line with Aslani et al., was recently conducted on a population from northeastern Brazil.[42] The investigators examined whether *VDR Fok*I polymorphism was associated with GDM risk in pregnant women who had spontaneous preterm births (SPTBs).[42] The results showed a significant association between carriers of the mutant *ff Fok*I genotype and the risk for GDM in SPTB patients compared to patients who had normal deliveries (OR = 4.71, CI 95% = 1.1–22.30; $P = 0.044$). The frequency of the *ff* genotype was higher in the SPTB group than their counterparts (23% vs. 4.7%). The authors suggested that *Fok*I VDR could be a biomarker for GDM.[42]

On the contrary, the third study, which was conducted in Saudi Arabia, did not show any significant association between VDR *Fok*I polymorphism and the risk of GDM when the investigators compared 112 GDM patients and 218 controls in their cross-

sectional study.[41] The previous findings of the studies should be interpreted cautiously. It should be stressed that El-Beshbishy et al.[41] and Aslani et al.[26] used fasting blood glucose levels alone to screen for GDM. However, the application of other criteria, such as the International Association of Diabetes and Pregnancy Study Groups' criteria, for the diagnosis of GDM results in an increased prevalence of GDM since it includes pregnant women with hyperglycemia.[43] Therefore, there might be a chance that the authors missed high-risk patients, and GDM incidence may have been under-reported and could have affected the study outcomes. It was also not clearly indicated in the previous studies if investigators controlled for risk factors such as obesity, a family history of diabetes, or a history of microsomia, as the latter two factors were significantly associated with GDM patients only in Aslani et al.'s study.[26]

To conclude, further studies with sufficient statistical power and on different ethnicities are warranted in order to confirm the potential of genetic biomarkers for the prediction of GDM in different populations. These prospective studies should take into consideration the inclusion of GDM risk factors while controlling for them to independently observe to what extent the *Fok*I VDR gene polymorphism is the culprit in increasing the risk of GDM.

2.2.1.3 *Fok*I VDR Gene Polymorphisms and Metabolic Syndrome

As mentioned in the previous chapter, MS is a serious health concern, and its prevalence is rising significantly in Saudi Arabia.[44,45] It is also more common in females than in males.[44] Disappointingly, despite the increasing prevalence of MS in Saudi Arabia, especially in women, no study to date has detected the role of the VDR gene *Fok*I

polymorphisms in the pathogenesis of maternal MS and its components in MS during pregnancy. However, a few studies have examined the association between VDR gene polymorphisms and MS in adults. Aslani et al., for example, found that the frequency of the *ff* genotype was twofold more frequent in postpartum Iranian women with MS when compared to the healthy participants during postpartum follow-ups ($P = 0.09$, OR = 2.1, 95% CI = 0.90–4.89).[26] On the contrary, another recent study from the same population in Iran by Shab-Bidar and colleagues did not observe any significant association between *FokI* and MS in adult males or females.[46] Other studies from Brazil,[47] north China,[48] and the United Arab Emirates are also in line with the previous findings. This evident variation and inconsistency between different studies on VDR *Fok*I polymorphism and MS could be attributed to environmental factors in different populations.[25] Moreover, the frequency distribution of the genotypes and alleles of the VDR gene polymorphisms differ substantially among different ethnic groups and within the same studied population, as previously noted in the Iranian population.[26,46] Such heterogeneity in the distribution of alleles and genotypes leads to different susceptibility to specific diseases.[49] Iyer et al. explained that in Saudi Arabia, genetic factors such as allele distributions and the exchange of gene pools through crossbreeding with neighboring countries can have a large effect on the findings of studies.[50] Accordingly, larger and more carefully designed prospective studies are needed that adjust for factors that might interfere with the underlying etiology of the disease.

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CHAPTER 3

PURPOSE OF THE STUDY

3.1 Research Aims and Hypotheses

As mentioned in **CHAPTER 2**, vitamin D receptor (VDR) polymorphism could be associated with increased risk of chronic disease resulting from defects in gene activation. The presence of the *FokI f* allele (mutant) has been shown to reduce transcriptional activity and affect vitamin D responsiveness. This, in turn, might lead to vitamin D deficiency and extra-skeletal health complications; yet, there is very limited research examining the association of the *FokI* VDR polymorphism with adverse pregnancy outcomes. The present research will investigate this association; particularly how maternal *FokI* VDR gene polymorphism affects metabolic health outcomes in pregnancy. The following chapter explains the aims and hypotheses of this research in greater detail.

Aim 1:

The first aim is to investigate the association between *FokI* VDR gene polymorphism and serum 25(OH)D levels in first-trimester pregnant Saudi women while controlling for lifestyle and environmental risk factors that are known to alter vitamin D status, such as dietary intake of vitamin D, sun exposure indices, physical activity, educational level, and place of residence.

Hypothesis 1:

We hypothesized that pregnant Saudi women carrying the *ff* genotype of the *FokI* VDR polymorphism will have an increased risk of vitamin D deficiency compared to

those carrying the *FF* and *Ff* genotypes.

Aim 2:

The second aim is to determine whether there was a difference in gestational diabetes mellitus (GDM) risk factors among the VDR *FokI* genotypes carried by pregnant Saudi women in their second trimester, via an oral glucose tolerance test (OGTT) administered at 24–28 weeks of pregnancy. We will also study the effect of different *FokI* VDR genotypes on glucose intolerance by measuring fasting blood glucose, fasting insulin, and glycated hemoglobin. We will also calculate Homeostasis model of insulin resistance and of beta cell function using HOMA-IR and HOMA_β, respectively.

Hypothesis 2:

We predicted that pregnant Saudi women carrying the *ff* genotype of the *FokI* VDR polymorphism will have increased glucose intolerance and a greater risk of GDM compared to those carrying the *FF* and *Ff* genotypes.

Aim 3:

The final aim is to assess whether the *FokI* VDR genotypes are associated with Metaboilc Syndrome (MS), or its components, in pregnant Saudi women. We will also study the metabolic impact of the *ff* genotype versus the wild *FF* genotype on various MS risk factors by measuring blood pressure, lipid and glycemic profiles, and body mass index (BMI).

Hypothesis 3:

We expect that pregnant Saudi women carrying the *ff* genotype of the VDR *FokI* polymorphism will be at higher risk of MS; elevated blood pressure; glucose

intolerance/gestational diabetes; dyslipidemia; and elevated lipid-profile biomarkers, such as total cholesterol, low-density lipoprotein (LDL), triglycerides (TG), and low high-density lipoprotein (HDL) cholesterol.

CHAPTER 4

MANUSCRIPT 1: THE INFLUENCE OF *FOKI* VITAMIN D RECEPTOR GENE POLYMORPHISMS ON VITAMIN D STATUS AMONG PREGNANT SAUDI WOMEN

4.1 Abstract

- **Background:** Vitamin D deficiency is highly prevalent among pregnant Saudi women, ranging from 86.4 % to 90.5%. It has been associated with adverse maternal and offspring outcomes. Numerous factors, including genetics, play significant roles in altering vitamin D status. Previous genomic studies have associated vitamin D receptor (VDR) gene polymorphism with serum 25-hydroxyvitamin D levels in adults. However, the genetic determinant of VDR gene polymorphisms and maternal vitamin D status in pregnancy have not been clarified yet.
- **Aim:** The objective of this study was to investigate the association between *FokI* VDR gene polymorphism and serum 25-hydroxyvitamin D levels in pregnant Saudi women in their first trimester, while controlling for lifestyle and environmental risk factors that are known to alter vitamin D status.
- **Materials and Method:** A cross-sectional study was conducted on 345 pregnant Saudi women who visited antenatal clinics of three hospitals in Riyadh, Saudi Arabia (King Khaled University Hospital [KKUH], King Salman bin Abdulaziz Hospital, and King Fahad Medical City [KFMC]) between December 2013 and January 2016. In the first antenatal visit, information was collected regarding socio-economic status and anthropometric and biochemical data, including serum 25 hydroxy-vitamin D

(25(OH)D) levels, dietary intake of calcium and vitamin D, physical activity, and sun exposure indices. The *FokI* VDR genotypes of each woman were determined by allele-specific polymerase chain reaction (PCR).

- **Results**: About 79% of the Saudi women studied were vitamin D deficient (25(OH)D < 50 nmol/L). Full body coverage with clothing, low physical activity, residence in North Riyadh, indoor work, and timing of sun exposure were found to be the key factors predisposing pregnant women toward vitamin D deficiency in the Kingdom of Saudi Arabia. In addition, the risk of being vitamin D deficient was associated with the *FokI* VDR genotype. The frequencies of genotypes *FF, Ff, ff,* and combined *Ff+ff* in women with vitamin D deficiency were 60.1%, 33.3%, 6.6%, and 39.9%, respectively, while in the non-deficient group they were 55.6%, 36.1%, 8.3%, and 44.4%, respectively. Taking the *FF* genotype as a reference, the *Ff* genotype and the combined variant genotype (*Ff+ff*) showed a significant decrease in the risk of developing vitamin D deficiency after adjusting for confounding factors (*FF* vs. *Ff*, OR = 0.44, 95% CI = 0.20–0.94, *P* = 0.035, *FF* vs. *Ff+ff*, OR = 0.42, 95% CI = 0.20–0.88, *P* = 0.022). Furthermore, individuals carrying the *f* allele were protected against vitamin D deficiency (OR = 0.51, 95% CI = 0.29–0.91, *P* = 0.021).
- **Conclusion**: Our study is the first to evaluate the association between *FokI* VDR gene polymorphism and vitamin D status in pregnant Saudi women while controlling for major risk factors. Our findings indicated that the *FF* genotype and the *F* allele of *FokI* VDR genotype polymorphisms is an important determinant of an individual's susceptibility to vitamin D deficiency in pregnant Saudi women after adjusting for confounding factors that have an effect on vitamin D status, such as age, BMI,

physical activity, full body clothing coverage, job status, residential area, and education.

4.2 Introduction

Vitamin D deficiency during pregnancy is of epidemic proportions, occurring in nearly 20% to 95% of pregnant women around the world.[1,2] There is a substantial body of evidence in the literature that indicate the serious adverse consequences of low maternal vitamin D status on maternal and fetal health, including both skeletal and non-skeletal outcomes.[3,4] Aside from the adverse skeletal effects, vitamin D deficiency during pregnancy has been linked to a number of maternal and fetal problems, including preeclampsia, gestational diabetes, caesarean section, bacterial vaginosis, postpartum depression, neonatal hypoglycemia, small-for-gestational age infants, and preterm birth.[1,5-8]

It is expected that the Saudi population will have adequate vitamin D status since cutaneous vitamin D production is primarily determined by sun exposure. Saudi Arabia has the highest number of sunny days per year, with an average sunlight of 2,200 kWh/m^2 and more solar radiation hitting the earth there due to the country's location within the earth's equatorial sun belt.[7] Unfortunately, numerous studies have demonstrated that vitamin D deficiency has become an epidemic among the Saudi population, specifically in the young and middle-aged groups of healthy Saudi adults, and in females more than males.[6,10] It is estimated that between 80–100% of Saudi women have suboptimal levels of vitamin D during the reproductive phase of their lives.[11-13] Higher prevalence of vitamin D deficiency among Saudi women appears to be a

predisposition for further deterioration of the vitamin D status, particularly among pregnant females because of the increased nutritional demands of the growing fetus.[14] Two recently published studies found that the prevalence of vitamin D deficiency was about 86% in pregnant Saudi women [10,15] and 88% among neonates.[15] Both of these studies, as well as other studies, used serum 25-hydroxyvitamin D (25(OH)D) < 50 nmol/L as a cut-off point for vitamin D deficiency.[10,15-17] Serum 25(OH)D is the main circulating metabolite of vitamin D and the most reliable biomarker of vitamin D adequacy,[18] reflecting both dietary vitamin D intake and cutaneous vitamin D production.[19]

Factors such as age, race, gender, skin pigmentation, physical activity, sun exposure, season, geographic location, latitude, obesity, and dietary/supplemental vitamin D intake have all been previously reported to influence serum 25(OH)D levels.[20,21] Other factors related to Saudi cultural practices and customs, including complete coverage of the body and limited outdoor activities play an important role in hindering the positive effect of the abundant sunlight on vitamin D status.[10]

Obviously, there are many factors that influence the levels of circulating 25(OH)D other than the ones mentioned earlier. Hussain *et al*. found that the prevalence of vitamin D deficiency was more common and severe in Saudis than in non-Saudis living in the same city of Riyadh, Saudi Arabia.[12] This observation might be explained by the influence of genetic variants such as vitamin D receptor polymorphisms that play a role in vitamin D metabolism and disease susceptibility.[10,20,22] In fact, genome wide association (GWA) studies have revealed that the genetic variation significantly contributes to variability in serum 25(OH)D concentration, with estimates of heritability

ranging from 29% to 80%.[23-25]

Vitamin D receptor (VDR) is one of the candidate genes involved in meditating the transcription of vitamin D-dependent target genes[26] by binding to the active hormonal form of vitamin D (1,25-dihydroxyvitamin).[27] VDR is known to be expressed in almost every tissue.[10] Several single nucleotide polymorphisms (SNPs) have been identified in the *VDR* sequence, including a *FokI endonuclease site* (rs10735810, merged into rs2228570).[28] The *FokI* VDR variant, which is located in an alternate upstream transcriptional initiation site of the VDR gene, specifically in exon 2 in the 5′-coding region, is the only known *VDR* polymorphism that is not linked to any of the other *VDR* variants. In addition, the VDR protein products differ with one variant (*f*) establishing an alternate start site resulting in a VDR protein that is 3 amino acids longer than the other shorter VDR variant (F). The longer f variant form of VDR has been shown to be less active in some in vitro test systems, thus giving it a potentially unique role. [24,29] *The FokI* mutation produces a longer *f*-VDR protein that is 427 amino acid long and a shorter *F*-VDR protein variant that is 424 amino acids long, which is reported to be more active and effective than the longer protein variant. [26] However, the functional significance of the *FokI* VDR polymorphism as a genetic risk factor in various disease outcomes is inconsistent.[30]

While there is increasing evidence in the literature associating *FokI* VDR gene polymorphisms with an increased risk of a range of adverse health outcomes, it is not yet clear whether the *FokI* VDR polymorphism contributes to maintaining serum 25(OH)D adequacy. The role of the *FokI* VDR polymorphism and other genetic variants related to vitamin D metabolic pathways have been greatly evaluated in regards to the responses of

serum 25(OH)D levels to vitamin D supplementation, with contradicting results.[31] The first study in the Saudi population that evaluated the genetic influence of *FokI* VDR as well as several single nucleotide polymorphisms (SNPs) in vitamin D-related genes on vitamin D status was reported by Sadat-Ali et al.[32] They found that *FokI* VDR was significantly associated with vitamin D deficiency, compared to the population with normal vitamin D levels.[32] Moreover, there are a limited number of studies that examined the effects of dietary vitamin D intake, sun exposure, or other personal characteristics as probable modifiers that influence serum 25(OH)D concentrations. [20] Additionally, most of the studies that examined such a relationship were conducted on groups of adults with different kinds of diseases. Such a correlation was not investigated among pregnant women in Saudi Arabia, where plentiful sunlight is available throughout the entire year. Therefore, we conducted a cross-sectional study to investigate the association between the *FokI* VDR gene polymorphism and serum 25(OH)D levels in pregnant Saudi women while controlling for environmental and lifestyle variables linked to Saudi culture that were previously known to influence vitamin D status.

4.3 Methods

4.3.1 Study Design and Sample Population

This study is part of a large prospective cohort study called "*Vitamin D and Pregnancy in Saudi Women*." Our cross-sectional study is a subset of these study participants, consisting of 345 pregnant Saudi women who visited an antenatal clinic at one of three different Saudi hospitals: King Khaled University Hospital (KKUH), King Salman Bin Abdulaziz Hospital, and King Fahad Medical City (KFMC) in Riyadh, the

capital of Saudi Arabia, between December 2013 and January 2016. The study has full ethical approval from the three hospitals to collect samples and patient data and approval from the Ethics Committee of the College of Science, King Saud University in Riyadh **(Appendix A).** Written informed consent was obtained from each patient **(Appendix B).**

4.3.1.1 Inclusion and Exclusion Criteria

Healthy pregnant Saudi women in their first trimester (8–12 weeks) aged 18 to 40 years, with no previous history of diabetes mellitus (type I or II), were recruited. Subject exclusion criteria included: non-Saudi subjects; gestational age of over 16 weeks; taking vitamin D supplements during pregnancy; unwillingness to deliver at any of the three hospitals; taking oral glucocorticoids; using drugs known to interfere with vitamin D or calcium absorption or parathyroid disorders; using any cardiac medication or diuretics; suffering from chronic hypertension or malabsorption syndrome; having chronic medical conditions or preexisting liver, kidney, calcium, and/or parathyroid conditions; or serious chronic disease conditions (epilepsy, cancer, or other malignancy).

4.3.1.2 Recruitment and Data Collection

Recruitment banners and brochures were placed in prenatal clinics in all three of the hospitals. Obstetricians were asked to introduce the research to their pregnant patients during their first prenatal appointment. Patients who met the criteria and consented to contribute to the present study were provided the appropriate information. During their early pregnancy visit, prospective candidates were asked to sign consent forms that included information about their participation in the study, such as the completion of a questionnaire regarding demographic data, procurement of blood samples for biomarker

measurement and DNA for genetic analyses, and anthropometric measurements. Permission for data collection from their medical records and stored blood stocks from a bio-bank were also obtained. Additionally, the participants were informed of their right to withdraw from the study at any point without it affecting their usual medical care.

The study tools included anthropometric measurements, blood biochemical tests, and an interview questionnaire. The interview questionnaire included questions regarding socio-economic information, clinical measurements, and past medical and treatment history. It also contained a food frequency questionaire (FFQ) and questions on sun exposure and physical activity.

4.3.2 Anthropometric Measurements

Anthropometric measurements were taken in the first antenatal visit. These measurements included weight (kg) and height (cm), used for calculating body mass index (BMI) (kg/m^2); reported pre-pregnancy weight (kg); and pre-pregnancy BMI (kg/m^2). Body weight, without shoes and in lightweight clothing, was measured to the nearest 0.1 kg with a stadiometer (Digital Pearson Scale, ADAM Equipment Inc., USA). Based on pre-pregnancy weight as self-reported during the prenatal visit, the pregnant women were classified according to the WHO BMI definitions as follows: underweight: < 18.5 kg/m^2; normal weight: 18.5–24.9 kg/m^2; overweight: 25.0–29.9 kg/m^2; or obese: $\geq$ 30.0 kg/m^2.[33] Height, to the nearest 0.5 cm, was measured during the early pregnancy visit using only (Digital Pearson Scale), with the women standing upright without shoes. Pre-pregnancy BMI was calculated from pre-pregnancy body weight recall and the measured height.

4.3.3 Interview and Physical Activity Questionnaire

The interview questionnaire was adapted from a previously published epidemiological survey in Saudi Arabia that included questions about socio-economic details, past medical and treatment history, and sun exposure **(Appendix C)**. [34-37] At the early pregnancy visit, all socio-economic and clinical measurements were recorded, including the visit date, age of the subject, place of birth, gestational age, maternal education status, occupation, and area of residence. Clinical data included date of LMP and estimated date of delivery (EDD). The participants were asked about parity (nulliparous or ≥ 1 multiparous). The season of the sampling was recorded. For the analysis of the season in relation to vitamin D status, the months of the year were divided into two periods. April to October was classified as summer, and November to March as winter.[38]

Furthermore, they were asked about sun exposure (yes/no) and other sun exposure indices such as exposure to the sun at work (indoor or outdoor work), timing of the exposure (e.g., noon), clothing (full body coverage vs. some parts of the body exposed), and the use of sunscreen (yes/no). Noon was defined in this study to be from 10:00 a.m. to 2:00 p.m.

Physical activity was also assessed through a well-known and validated questionnaire, namely an Arabic short version of an International Physical Activity Questionnaire (IPAQ).[39] The short IPAQ has been validated in various studies among different populations, including pregnant subjects.[40,41] IPAQ evaluates physical activity during periods of leisure, work, and commute. It also assesses the intensity of an activity as vigorous, moderate, low-intensity physical activity/walking, or sitting. Each subject

was assisted in recalling her physical activities over the previous week. For each type of activity, frequency and duration were recorded in days per week and minutes or hours per day. This study excluded moderate and high activity because few women carried out such activities during pregnancy. The present study analyzed low-intensity physical activity and minutes spent sitting per week, similar to other studies using IPAQ in pregnancy.[42] Low-intensity physical activity in minutes per week was applied as a continuous and categorical variable at either less than or over 210 minutes/week.[43]

4.3.4 Dietary Data Collection and Processing

The dietary tool applied for measuring the participants' food intake was the FFQ, which has been used previously in Saudi Arabian studies. [35,37] The subjects were interviewed separately, and the data were gathered by applying a pre-designed questionnaire to measure aspects of food consumption among the expectant mothers over the course of one week. The main purpose of this was to assess calcium, vitamin D, fat, and protein intake, as well as any other nutrients that may affect the absorption or excretion of vitamin D and calcium. Responses to the dietary questionnaire were facilitated through food models, cups and spoons of various sizes, cans, and approximate portions, using hand gestures to help the participants recall the amount of food consumed.

The FFQ consisted of eight parts pertaining to: (1) bran, starch, and grains; (2) meat and fish; (3) fats and oils; (4) dairy products; (5) fruits and vegetables; (6) traditional dishes; (7) sweets and soft drinks; and (8) coffee and tea. Starches and grains mainly referred to any bran food that could have reduced calcium absorption.[44] Fruits and vegetables included those high in calcium and any that may have reduced calcium absorption.[45] Dairy fats (whole fat, low fat, skim milk, or other products) and fat added

during cooking may also affect calcium absorption.[46] Finally, data on supplement intake (yes/no) were collected, including multivitamins, folic acid, iron, vitamin D, and cod liver oil.

Nutrient intake was calculated using U.S. Department of Agriculture (USDA) software (27th edn, 2014), along with Nutribase software (11th edn, 2014), which utilizes food macro- and micro-nutrient composition. For traditional Saudi food, an Arabic food analysis program was used (1st version, 2007). The assessment of everyday food intake was conducted as total intake of macro- and micro-nutrients, especially vitamin D and calcium. Dietary nutrient values were compared with the dietary reference intake (DRI) of micro-nutrients during pregnancy, such as vitamin D and calcium.[47] Additionally, vitamin D and calcium intake were presented continuously and categorically as above 600 IU/day and 1,000 mg/day of vitamin D and calcium, respectively.

4.3.5 Biochemical Assessment

Blood samples (10 ml) were collected using a sterile vacutainer blood collection apparatus. Whole blood, serum, and ethylene diamine tetra-acetic acid (EDTA) plasma were collected from the participants. All samples were aliquoted and stored in a freezer at -80 ^{o}C to facilitate their availability for subsequent chemical analysis. All the lab tests were performed on the serum. The plasma was stored if needed for further analysis. The samples were stored and analyzed in the *Biomarkers Research Program* (*BRP*) laboratory.

4.3.5.1 Primary Outcome Measures

Our main outcome measure was to investigate the association between *FokI* VDR gene polymorphism and serum 25(OH)D levels in pregnant Saudi women while controlling for confounding factors, which affect vitamin D status.

4.3.5.2 Assessment of Circulating 25(OH)D and Biochemical Parameters

During the first antenatal visit, random blood samples were collected to measure total 25(OH)D, calcium, phosphorus, and alkaline phosphatase. Total 25(OH)D was measured in an ECLIA assay, (Cobas e 411; Roche Diagnostics GmbA, Mannheim, Germany). The BRP laboratory is a participating entity in the Vitamin D External Quality Assessment Scheme (DEQAS). Since no international consensus exists for vitamin D deficiency cut-off points during pregnancy, vitamin D was categorized according to 25(OH)D concentrations as follows: < 50 nmol/L considered to be a deficiency and ≥ 50 nmol/L considered to be non-deficient.[16,17]

Serum calcium and phosphorous were measured using a routine laboratory chemical analyzer (Konelab, Finland). Meanwhile, bone-specific alkaline phosphatase (BAP) was measured using the LIAISON XL immunoassay (DiaSorin, Italy).

4.3.5.3 DNA Extraction and Quantification

Genomic DNA was extracted from whole blood using innuPREP blood mini kits (Analytik Jena, Germany) by following the manufacturer's instructions. In brief, 200 µl of the whole blood sample was added to a 1.5 ml reaction tube containing lysis buffer and Proteinase K. The contents were mixed using a vortex and incubated at 60 ^{0}C for 10 minutes. The required amount of binding solution was added to the tube and transferred

to a spin filter column with a receiver tube and centrifuged for 1 minute at 12,000 rpm. The column-bound DNA was washed using the kit's washing solution C, followed by washing solution BS. As a final step, the spin filter was placed in a 1.5 ml elution tube, and 200 µl of elution buffer that was pre-warmed to 60 ^{0}C was added. After centrifugation, the collected DNA was stored at -20 ^{0}C for further analysis. DNA concentration and purity (260/280) was determined using a Nano-Drop spectrophotometer.

4.3.5.4 *FokI* VDR SNP Genotyping

The *FokI* SNP (rs 2228570), previously reported as (rs10735810)[20] (Exon 2, C[*F*] > T[*f*]), *is* located in the VDR coding region and can be evaluated by allelic discrimination real-time PCR using pre-designed TaqMan genotyping assay from Applied Biosystems, Foster City, CA, USA (assay ID: C_12060045_20). Amplification reactions were performed in a volume of 10 µL containing 1X TaqMan genotyping Master Mix (Applied Biosystems), 1X mix of unlabeled PCR primers and TaqMan MGB probes, and 30 ng of template DNA. All amplifications and detections were conducted in 96-well PCR plates using a Bio-Rad CFX96 Real-Time PCR Detection System (Bio-Rad, Milan, Italy). Thermal cycling was initiated with a denaturation step of 10 min at 95 °C, followed by 45 cycles of 15 s at 95 °C and 90 s at 60 °C. After PCR was completed, allelic discrimination was analyzed using the Bio-Rad CFX Manager Software (Version 1.6, Bio-Rad). Genotype assignment was determined by plotting the end point relative fluorescent units (RFU) for one fluorophore (allele 1 on the x-axis) against the RFU for the other fluorophore (allele 2 on the y-axis) on the allelic discrimination. All PCR reactions were set up in a dedicated PCR area with dedicated PCR pipettes and reagents.

4.3.6 Statistical Analysis

The sample size was estimated for adequate statistical power. The significant differences in serum 25(OH)D levels according to *FokI* VDR genotype were based on an expected effect size of 0.25,[48] and the required sample size at 95% CI was 243, with 95% power.

The data were analyzed using SPSS version 21.0 statistical software (SPSS, Chicago, IL, USA). Quantitative normal variables were expressed as mean ±SD, while quantitative non-normal variables were expressed as medians (Q1-Q3). Categorical variables were expressed as frequencies and percentages (%). Pearson's chi-square test was used to determine the differences between categorical variables. Comparisons between groups were performed using the independent sample t-test and the Mann-Whitney U test for normal and non-normal variables, respectively. Unadjusted and adjusted odds ratios were determined using logistic regression analysis. P-value < 0.05 is considered significant.

4.4 Results

4.4.1 Demographics and Biochemical Characteristics According to Vitamin D Status in Early Pregnancy

Table 4.1 presents the characteristics of the deficient and non-deficient groups of pregnant women. The population sample was categorized into either deficient (< 50 nmol/L) or non-deficient (≥ 50 nmol/L) groups for statistical analysis. [49,50] The prevalence of vitamin D deficiency (< 50 nmol/L) in early pregnancy was 79.1% (273/345).

The mean age of women with vitamin D deficiency was similar to that of non-deficient women (28.7 ± 5.4 vs. 29.4 ± 5.4 years, $P = 0.320$). Pre-pregnancy BMI, BMI in early pregnancy, and parity also failed to reveal any differences between the two groups. The biochemical profile of the pregnant women, such as calcium and alkaline phosphatase levels, did not show any significant differences between the groups. However, the non-deficient subjects had significantly higher serum phosphorus and 25(OH)D levels than the deficient subjects, (1.3 ± 0.5 vs. 1.2 ± 0.4 mmol/L, $P = 0.029$) and (67.8 ± 12.9 vs. 27.0 ± 10.6 nmol/L, $P < 0.001$), respectively.

Interestingly, the percentage of women engaged in low-intensity physical activity such as walking was significantly higher in the non-deficient group than in the deficient group (78% vs. 43.8%, $P < 0.001$), respectively. Furthermore, pregnant women who were non-deficient in vitamin D were more likely to be educated and have a graduate or postgraduate degree than those in the deficient group (70.4% vs. 56.0%, $P = 0.028$), respectively. Moreover, the rate of employment was higher in the non-deficient group compared to the deficient group, although this was not statistically significant (38.9% vs. 29.5%, $P = 0.127$). Similarly, vitamin D, calcium, and multivitamin intake did not reveal any statistically significant differences between the deficient and non-deficient groups.

The percentage of women living in North Riyadh was significantly higher amongst the non-deficient subjects compared to their counterparts (33.3% vs. 15.9%, $P < 0.001$). Meanwhile, a higher percentage of pregnant women who were deficient in vitamin D lived in West Riyadh compared to the non-deficient group, but this figure did not reach statistical significance (18.8% vs. 29.8%, $P = 0.069$).

We did not observe any differences between vitamin D non-deficient and deficient groups in exposure to sun, use of sunscreen, and blood being drawn in the summer or winter. Nevertheless, the amount of exposure to the sun at noon was significantly higher amongst the non-deficient subjects compared to their deficient counterparts (72.2% vs. 22.7%, $P < 0.001$), respectively. Likewise, the percentage of women who tend to work indoors was higher amongst the deficient participants compared to the non-deficient subjects, (98.5% vs. 66.7%, $P < 0.001$), respectively. Moreover, the percentage of subjects covering their whole body with clothing was significantly higher in the deficient group compared to their counterparts, (33.3% vs. 2.8%, $P < 0.001$), respectively.

4.4.2 Risk Factors for Vitamin D Deficiency in Pregnancy

We further conducted a multivariate logistic regression analysis to assess the risk factors for vitamin D deficiency while adjusting for confounding factors (**Table 4.2**). Significant independent variables that increased the risk of vitamin D deficiency included full body coverage with clothing (OR 19.64, 95% CI 2.52-152.68, $P = 0.004$), and working indoors (OR 132.0, 95% CI 10.9-1600.7, $P < 0.001$).

On the contrary, the variables that appeared to confer some protection against vitamin D deficiency in early pregnancy were higher serum phosphorus levels (OR 0.27, 95% CI 0.10-0.76, $P = 0.013$), low-intensity physical activity/walking (≥ 210 minutes/week) (OR 0.19, 95% CI 0.08-0.44, $P < 0.001$), time of sun exposure at noon (OR 0.13, 95% CI 0.06-0.32, $P <0.001$), and living in North Riyadh (OR 0.32, 95% CI 0.14-0.75, $P = 0.009$).

Moreover, having a graduate or bachelor's degree, and a higher serum calcium levels (OR 0.42, 95% CI 0.17-1.02, *P* = 0.054), (OR 0.11, 95% CI 0.01-1.18, *P* = 0.068), respectively, appeared to be protective factors against vitamin D deficiency, but did not reach significant levels.

4.4.3 *FokI* VDR Gene Polymorphism and Vitamin D Deficiency

As shown in **Table 4.3**, the difference in the occurrence of the various *FokI* VDR genotypes in vitamin D deficient and non-deficient participants was statistically significant. The *FokI* VDR genotypes are normally expressed as homozygous genotype "*FF*," heterozygous genotype "*Ff*," and mutant homozygous genotype "*ff*." The frequencies of genotypes *FF, Ff, ff,* and combined *Ff+ff* in women with vitamin D deficiency were 60.1%, 33.3%, 6.6%, and 39.9% respectively, while in the non-deficient group they were 55.6%, 36.1%, 8.3%, and 44.4%, respectively. We did not observe any statistically significant associations between *FokI* VDR polymorphisms and serum 25(OH)D levels, but after adjusting for important covariates such as age, BMI, low-intense physical activity, full body clothing coverage, job status, residential area, and education, we detected a statistically significant association between VDR genotype and risk of vitamin D deficiency.

After correcting for the potential confounding variables, and using the *FF* genotype as a reference, the *Ff* genotype and the combined variant genotype (*Ff+ff*) showed a significant decrease in the risk of developing vitamin D deficiency (*FF* vs. *Ff*, OR = 0.44, 95% CI = 0.20–0.94, *P* = 0.035; *FF* vs. *Ff+ff*, OR = 0.42, 95% CI = 0.20–0.88, *P* = 0.022). Furthermore, individuals with the *f* allele were protected against vitamin D deficiency (OR = 0.51, 95% CI = 0.29–0.91, *P* = 0.021). However, no significant

association was found between the *ff* genotype and vitamin D deficiency (*FF* vs. *ff*, OR = 0.36, 95% CI = 0.08–1.52, *P* = 0.163).

4.5 Discussion

In our study, vitamin D deficiency was evident in 79.1% of our study sample. Although numerous studies have reported a high prevalence of vitamin D deficiency in the Saudi population across different ages, [35, 51,52] only a limited number of studies have assessed the prevalence of vitamin D deficiency in pregnant Saudi women. [6,10,15, 53] For example, Al-Ajlan et al. found that 68% of Saudi pregnant women were vitamin D deficient.[53] Al Foda et al. [15] and Al-Sheikh et al.[10] found a higher prevalence of vitamin D deficiency reaching 86%.[10,15] Besides Saudi Arabia, vitamin D deficiency during gestation seems to be a global health concern, as it has been reported in many other countries such as the UAE (78%),[54] India (62%),[55] Korea (77.3%),[56] Belgium (44.6%),[57] Switzerland (63%),[58] and Canada (39%).[59]

Despite the abundant sunshine all year in Saudi Arabia, our results indicated that lifestyle patterns and traditional clothing play an important role in decreasing sun exposure, thus significantly decreasing vitamin D levels and the risk of vitamin D deficiency. Saudi women wear black veils, or what are known as *abaya*, to cover their entire bodies due to religious and cultural beliefs. The present study, supported by others, indicates that pregnant women who fully cover their body are at greater risk of developing vitamin D deficiency. [11,60]

Moreover, we found higher levels of vitamin D among educated pregnant women compared to their less educated counterparts. This observation has been stated in different studies reporting that vitamin D levels vary in adults according to their

educational acheivement, with a higher level of education decreasing the risk of vitamin D deficiency.[61,62]

We also noticed that the area of residence, particulary North Riyadh, had a significantly positive impact on vitamin D status compared to other areas. In other words, pregnant women residing in North Riyadh had higher serum 25(OH)D levels than women residing in other areas such as south, west, and east of Riyadh. We speculate that this part of the capital city is more likely to be affluent and has highly educated residents.

Pregnant women performing low-intensity physical activity such as walking were also shown to have higher vitamin D levels compared to those performing low-intensity physical activity in the deficient group. Similarly, Al-Faris et al. also noted that pregnant women involved in daily outdoor activity during the early stages of pregnancy were more likely to have adequate vitamin D levels.[6] These observations may be attributed to two reasons. First, increased physical activity causes greater mobilization of vitamin D from burned fat deposits.[63] Second, engaging in an outdoor asctivity means greater sun exposure, thus triggering vitamin D synthesis. [64]

In our present study, we did not detect any significant associations between vitamin D levels and the season in which the blood was drawn from the samples when comparing the deficient and non-deficient pregnant women. However, this result was expected due to several reasons. Firstly, women tend to cover their whole bodies with clothing due to cultural and religious reasons.[10] Secondly, due to the extreme hot weather in the summer, Saudis generally avoid sun exposure.[6,53] Thirdly, a large proportion of the workforce works indoors in Saudi Arabia. The latter two explanations confirm our finding that working indoors and the amount of sun exposure, especially at noon, were

also critical in determining vitamin D levels and corresponded significantly with risk of having vitamin D deficiency in our population. Our results were in line with the findings of Al-Shahrani et al.[65] They reported that the time of day plays a major role in vitamin D production, as they observed that summer production of pre-vitamin D_3 increased between the peak hours of 10:00 a.m. to 12:00 p.m.[65]

We did not observe a significant difference in dietary intake of vitamin D and calcium between deficient and non-deficient participants, as they were shown to be very low in both groups in our study, as well as in other studies conducted in Saudi Arabia.[6,66] In the present study, we found that about 25% of the entire sample were taking multivitamins in their first trimester. Consistently, other studies from various countries have reported the use of multivitamin supplementation during pregnancy, ranging from 7.5% to 63.1%. [6,57,67]

It has been previously noted that vitamin D intake during pregnancy, whether from food or from supplements, is inadequate for meeting the optimum demands of the mother and the growing fetus.[68] Previous studies have reported that less than 10% of the daily vitamin D requirement could be met through dietary sources of vitamin D.[69,70]

In Saudi Arabia, dietary sources of vitamin D tend to be inadequate, and currently food fortification is extremely scarce.[70] In addition, globalization has brought dramatic changes to dietary and lifestyle patterns, with a shift toward unhealthy diets.[6,60] Since our study was conducted in the capital city of Riyadh, the consumption of vitamin D-rich fish was usually uncommon due to the geographic nature of the area, which lacks access to the sea or rivers.[11]

Interestingly, in our study we observed the novel finding of an association between *FokI* VDR gene polymorphism and the risk of vitamin D deficiency in pregnant Saudi women after adjusting for lifestyle and environmental factors that are known to alter vitamin D levels. Our results showed a significant difference in the genotype distribution between vitamin D deficient and non-deficient pregnant women. Our results indicated that *FF* genotype individuals have a significantly higher risk for vitamin D deficiency compared to those carrying the *Ff* genotype and the combined genotype (*Ff+ff*). Based on our data, subjects carrying the *FokI F* allele had a significantly higher prevalence of vitamin D deficiency than subjects with *f* alleles.

There is consistent evidence that *FokI* VDR gene polymorphism is significantly associated with 25(OH)D levels in adult patients with Multiple Sclerosis (MS),[18,71,72] Systemic Lupus Erythematosus (SLE),[73] Ulcerative Colitis,[29] Crohn's Disease,[79] and Autism Spectrum Disorder (ASD) in children.[48]

Three studies investigated the association between *FokI* polymorphism and serum 25(OH)D levels in MS patients and found results that were similar to our findings. Agnello et al. observed that MS patients with the *ff* genotype had significantly higher levels of serum 25(OH)D compared to *FF* and *Ff* carriers.[71] In addition, Orton et al. reported the same finding in a study on twins with MS.[18] Similarly, another study by Smolders et al. demonstrated that lower serum 25(OH)D levels were associated with the homozygous *FF* genotype compared to the homozygous *ff* genotype carriers.[72] Another study conducted in Turkey by Coskun et al. reported that the *ff* genotype was associated with higher serum 25(OH)D levels in children with ASD compared to the homozygous *FF* genotype.[48] In addition, two studies conducted in patients with SLE living in Brazil[73]

and Egypt[74] found that carriers of the wild type homozygous *FF* genotype in comparison with the mutant homozygous *ff* genotype had significantly lower serum 25(OH)D.

Intriguingly, Yao et al. conducted a randomized double-blinded, placebo-controlled trial and found that carriers of the *f* allele responded better to vitamin D treatment (2000 IU/d) than *F* allele carriers in the Chinese population with vitamin D deficiency.[27] In fact, when compared to other common polymorphisms in vitamin D metabolism genes, *ff FokI* genotype was associated with a greater increase in 25(OH)D in week 20 of the treatment, but the treatment was still unable to correct vitamin D deficiency in 25% of participants who were carrying other mutant genes. [27]

These results, as well as our findings regarding the mutant *f* allele's protective role against vitamin D deficiency should be interpreted cautiously. Monticielo et al. explained that the underlying justification for such an observation could be that the mutant *f* allele may be associated with VDR dysfunction since it is longer and less active than the *F* allele, thus causing an increased synthesis of serum 25(OH)D from vitamin D. [73] However, there are definitely other described genetic factors along the vitamin D metabolic pathway that influence the production, elimination, and transportation of serum 25(OH)D concentration, which we did not measure and may contribute to residual confounding in our study.

If we delve into the underlying mechanism of vitamin D metabolism, we notice that 1,25-dihydroxyvitamin D3 (1,25$(OH)_2$D3) binds to the *VDR*, and the activated *VDR* regulates the rate of transcription of vitamin D-responsive genes.[75] VDR-1,25(OH)2 D complex is considered a negative regulator of vitamin D-25-hydroxylase enzyme (also known as CYP27A1), which catalyzes the 25-hydroxylation of vitamin D. [71]

Furthermore, it is well known that *FokI* VDR polymorphism can alter the translation initiation sites of the VDR gene.[48,71] The short VDR variant (*F* allele) is associated with a higher transcriptional activity than the long variant (*f* allele).[48] According to this evidence, the authors attributed the observation of higher vitamin D levels in individuals with the *ff* genotype to the following explanation: Since the *F* allele variant of the FokI VDR genotype is more active, it is more likely to enhance the inhibitory effect of the VDR on CYP27A1 than the *f* allele, causing reduced serum 25(OH)D levels. [71,72]

Interestingly, there are other studies that have reported an association between *FokI* VDR polymorphism and vitamin D status, but their findings contradict ours. These studies show that the *ff* genotype could be a risk factor for vitamin D deficiency based on the fact that this mutant genotype could alter the metabolic feedback loops or affect the speed at which 25(OH)D is metabolized.[18] Thus, individuals carrying the homozygous major genotype *FF* have a significantly better increase in serum 25(OH)D levels than those who carry the *ff* genotype.[76] This assumption led Al-Daghri and colleagues to speculate that *FokI* genotypic variants might require different threshold concentrations of 25(OH)D levels to exhibit feedback effects. The following section will shed light on the studies that contrast our findings.

Two studies conducted in Egypt found a significant association between *ff* genotype carriers and low values of serum 25(OH)D when compared to *FF* genotype in type 2 diabetic (T2DM) patients.[77,78] In agreement with the previous results, two studies conducted in China also evaluated the association between *FokI* VDR polymorphism and vitamin D status among patients with *inflammatory bowel disease* (*IBD*) and found similar outcomes.[79,80] Both studies demonstrated a significant association between

vitamin D deficiency and the combined mutant genotype (*Ff+ff*) of *FokI* in the Chinese patients with ulcerative colitis (UC)[29] and Crohn's Disease (CD).[79] Moreover, the investigators observed a higher risk of UC [29] and CD [79] in patients when *FokI* VDR mutations and vitamin D deficiency were combined. Similarly, a very recent cross-sectional study conducted among black and white South Africans found an increased risk of developing severe vitamin D deficiency (< 38 nmol/L) among chronic kidney disease (CKD) patients *carrying Ff* and combined *Ff+ff* genotypes compared to healthy participants.[80]

Furthermore, two vitamin D supplementation studies conducted on type 2 diabetic (T2DM) patients reported that the lowest increase in serum 25(OH)D levels was detected in patients with *ff* genotypes.[26,76] With that knowledge, Al Daghri et al. [76] supplemented their patients with 2,000 IU vitamin D3 daily for 12 months without a control or placebo group, while Neyestani et al. monitored the daily intake of vitamin D-fortified yogurt with 500 IU vitamin D/250 mL for 12 weeks.[26] Both authors suggested that high-risk patients carrying *ff* genotype might be considered "low responders," as they need higher doses of vitamin D supplements than their counterparts in order to achieve sufficient levels and benefit the most from the treatment.[26,76]

To our knowledge, only one study has explored the effect of *FokI* VDR polymorphism on serum 25(OH)D levels in healthy Saudi subjects.[32] Sadat-Ali et al. found that vitamin D deficiency was quite prevalent among 283 Saudis, reaching 87%, and that carriers of the GG genotype of *FokI* VDR (which corresponds to *FokI ff* genotypes in our study) had an increased risk of both vitamin D insufficiency and deficiency when compared to controls, [32] a study that contradicts ours. The investigators

addressed a major limitation in their study that they did not control for confounders such as environmental and individual factors that may alter vitamin D levels. [32] It is worth mentioning that we did not see any significant associations between *FokI* VDR polymorphism and vitamin D deficiency in our logistic regression model until we adjusted for risk factors such as age, BMI, physical activity, clothing, job status, residential area, and education level. It seems that these factors have masked the genetic influence on vitamin D status, and this could be the case with Sadat Ali et al.'s findings as well.

While there is robust evidence linking *FokI* VDR polymorphism to serum 25(OH)D concentrations, many studies have failed to replicate these finding in different populations. Neither Li et al. [81] nor Robein et al. [20] found a significant association between *FokI* VDR gene polymorphism and vitamin D deficiency in the Chinese population, even though Robein et al. [20] controlled for confounding factors such as dietary vitamin D intake and number of hours spent indoors at work. Also, Engelman et al. did not observe any effect of *FokI* on vitamin D levels in the African-American or Hispanic populations.[82] Similarly, no association was detected among Saudi [83] and Emirates [84] participants with Metabolic Syndrome (MS).

Although there is a vast body of literature stating that pregnancy offers a window of opportunity to prevent adverse fetal programming, we only found one study that evaluated the association between *FokI* VDR polymorphism and vitamin D levels in Iranian pregnant women. [85] Aslani et al. did not detect any significant differences in 25(OH)D levels between carriers of the *F* and *f* alleles among healthy pregnant women

and gestational diabetes mellitus (GDM) patients in their second trimester (17.27 ± 13.56 vs. 16.86 ± 9.79 nmol/L, $P = 0.7$). [85]

Possible explanations for the non-significant association between VDR *FokI* and vitamin D status in the previous studies could be attributed to the following reasons: smaller study populations and the failure to adjust for risk factors that alter serum 25(OH)D, such as vitamin D intake, use of supplements, latitude, seasonal variations, sun exposure, and other factors that affect vitamin D status. [20] Additionally, most of the research to date has assessed the association between 25(OH)D and *FokI* VDR on non-healthy subjects, and the lack of association could be related to disease state confounders. To the best of our knowledge, this is the first investigation to assess VDR *FokI* polymorphism and vitamin D levels in a group of pregnant women with prevalent vitamin D deficiency in Saudi Arabia. We also extensively studied important confounders that have a significant effect on altering vitamin D status.

We acknowledge that there are some limitations to the current study. First, the cross-sectional nature of the study cannot confirm a causal link between vitamin D levels and VDR *FokI*, and further prospective design studies are required to support our findings. Second, we did not study polymorphisms of other *VDR* genotypes and other genes involved in the vitamin D metabolic pathway such as Vitamin D-binding protein, Vitamin D 25-hydroxylase, and CYP27A1 that are thought to be associated with vitamin D status.[31] Third, despite several adjustments, factors related to sun exposure and the use of sunscreen were subjected to recall bias.

Despite the aforementioned limitations of this study, the finding in our study may assist in identifying “at-risk” populations through screening for *FokI* VDR

polymorphisms in conjunction with screening vitamin D serum levels in pregnant women. Moreover, since vitamin D is a potentially modifiable risk factor, these results encourage a prospective study of the use of vitamin D supplementation in pregnant women who are vitamin D deficient and the monitoring of its effectiveness in increasing vitamin D levels while evaluating genes related to the metabolic pathway.

4.6 Conclusion

As far as we know, this is the first cross-sectional study to evaluate the association between *FokI* polymorphisms in the *VDR* gene and serum 25(OH)D levels in pregnant Saudi women with vitamin D deficiency compared to non-deficient subjects. We also demonstrated the first association between the *f* allele and the reduced risk of vitamin D deficiency when controlling for lifestyle and environmental factors that play a major role in altering vitamin D status. The observed higher levels of serum 25(OH)D in *f* allele carriers may be a compensatory response due to reduced VDR activity and distorted signaling pathways.

In conclusion, vitamin D deficiency was highly prevalent among 79% of pregnant Saudi women in their first trimester. Full body coverage with clothing, lower levels of physical activity, residence in areas other than North Riyadh, work indoors, and the amount of exposure to sunlight were found to be major risk factors for vitamin D deficiency in pregnant Saudi women.

Our findings also indicated that the VDR gene *FokI FF* genotype and the *F* allele might be important determinants of an individual's susceptibility to vitamin D deficiency in pregnant Saudi women, thus leaving them unprotected with the risk of developing adverse skeletal and non-skeletal health outcomes. However, allele *f* and genotype *Ff*

seemed to play a protective role, but the role of genotype *ff* is still unclear because of its limited frequency.

It is important to take this finding into consideration in future prospective studies that are targeted to assess 25(OH)D levels. Therefore, possible alterations in vitamin D intake are needed to ameliorate the untoward effects of VDR *FokI* polymorphisms on vitamin D status in this vulnerable vitamin D deficient group carrying a variant of the *F* allele. If genotypes are truly associated with vitamin D status, serum concentrations of 25(OH)D required to reduce adverse health outcomes may need to be individualized for each patient.

Future studies on healthy subjects are required to clarify the primary genetic predictors of 25(OH)D concentrations, including the assessment of genes involved in vitamin D metabolism. However, our study indicates the need for proper adjustment for important confounders that are known to alter vitamin D status to appreciate the effects of genotype on vitamin D levels.

Table 4.1: Demographic and biochemical characteristics of vitamin D deficient vs. non-deficient subjects

Parameters	Overall	Non-Deficient ≥50nmol/L	Deficient <50 nmol/L	*P*-values
N	345	72 (20.9)	273 (79.1)	---
Age (years)	28.8 ± 5.4	29.4 ± 5.4	28.7 ± 5.4	0.320
BMI (kg/m^2)	28.2 ± 6.3	28.6 ± 6.6	28.2 ± 6.3	0.626
Pre-pregnancy BMI (kg/m^2)	27.2 ± 6.1	27.5 ± 6.7	27.1 ± 5.9	0.591
Parity #	1.4 ± 1.7	1.3 ± 1.7	1.4 ± 1.7	0.492
Biochemical Parameters				
Calcium (mmol/L)	2.2 ± 0.2	2.2 ± 0.2	2.2 ± 0.2	0.638
Phosphorus (mmol/L)	1.2 ± 0.4	1.3 ± 0.5	1.2 ± 0.4	0.029
Alkaline phosphatase (mmol/L)	9.5 ± 3.2	9.5 ± 2.8	9.5 ± 3.3	0.992
25(OH) D (nmol/L)	35.5 ± 20.0	67.8 ± 12.9	27.0 ± 10.6	0.000
Physical Activity				
Low-intense PA (<210 mint/wk) (yes)	144 (50.9)	46 (78.0)	98 (43.8)	<0.001
Sitting (minutes/week)	1200(600-1800)	1200 (450 – 1500)	1200 (600 – 1800)	0.709
Diet				
Vitamin D intake (>600 IU/d)	10 (3.1)	4 (5.8)	6 (2.4)	0.433
Calcium intake (>1000 mg/d)	21 (6.6)	6 (8.7)	15 (6.0)	0.147
Multivitamin intake	62 (21.9)	13 (24.5)	49 (21.3)	0.609
Education and employment				
University graduate (or Higher) (yes)	199 (59.1)	50 (70.4)	149 (56.0)	0.028
Employment (yes)	107 (31.5)	28 (38.9)	79 (29.5)	0.127
Area of living				
Living in north Riyadh (yes)	64 (19.6)	23 (33.3)	41 (15.9)	<0.001
Living in west Riyadh (yes)	90 (27.5)	13 (18.8)	77 (29.8)	0.069
Sun exposure Indices				
Blood sample collection season	111 (32.4)	23 (32.9)	88 (32.2)	0.921

(Summer)				
Sun exposure (yes)	91 (26.4)	20 (27.8)	71 (26.0)	0.762
Coverage with clothing (whole body)	93 (27.0)	2 (2.8)	91 (33.3)	<0.001
Use of sun screen (yes)	28 (8.1)	6 (8.3)	22 (8.1)	0.939
Nature of work (indoors)	317 (91.9)	48 (66.7)	269 (98.5)	<0.001
Time of sun exposure (at noon)	114 (33.0)	52 (72.2)	62 (22.7)	<0.001

Note: Data presented as Mean ± SD for continuous variables and N (%) presented for categorical variables;
# indicates non-normal variables; *P*-value<0.05 considered significant.

Table 4.2: Predictors of vitamin D deficiency among pregnant women in early pregnancy

Parameters	Univariate Analysis		Adjusted Model	
	OR (95% CI)	*P*-value	OR (95% CI) *	*P*-value*
Age (years)	0.98 (0.93 – 1.02)	0.319	1.00 (0.92 – 1.08)	0.909
BMI (kg/m^2)	0.99 (0.95 – 1.03)	0.625	0.97 (0.92 – 1.03)	0.288
Pre-pregnancy BMI (kg/m^2)	0.99 (0.55 - 1.03)	0.590	0.97 (0.91 – 1.03)	0.283
Parity	1.06 (0.90 – 1.25)	0.491	1.13 (0.85 – 1.50)	0.403
University graduate or postgraduate	0.54 (0.30 - 0.94)	0.030	0.42 (0.17 – 1.02)	0.054
Employment	0.66 (0.38 – 1.13)	0.128	0.67 (0.31 – 1.47)	0.319
Living in North Riyadh	0.38 (0.21 - 0.69)	0.002	0.32 (0.14 - 0.75)	0.009
Living in West Riyadh	1.35 (0.97 - 1.88)	0.072	1.42 (0.91 – 2.22)	0.119
Calcium (mmol/L)	0.70 (0.16 – 3.02)	0.637	0.11 (0.01 – 1.18)	0.068
Phosphorus (mmol/L)	0.42 (0.21 – 0.83)	0.012	0.27 (0.10 – 0.76)	0.013
Alkaline phosphatase (mmol/L)	1.00 (0.92 – 1.09)	0.992	0.96 (0.83 – 1.11)	0.596
Low-intense PA (<210 mint/wk) (yes)	0.22 (0.11 – 0.43)	<0.001	0.19 (0.08 – 0.44)	<0.001
Vitamin D intake (>600 IU/d)	0.40 (0.11 – 1.46)	0.165	0.30 (0.02 – 4.52)	0.383
Calcium intake (>1000 mg/d)	0.67 (0.25 – 1.80)	0.427	2.94 (0.30 – 28.35)	0.351
Blood collection season (Summer)	0.97 (0.56 – 1.70)	0.921	2.49 (0.90 – 6.89)	0.080
Sun exposure (yes)	0.91 (0.51 – 1.64)	0.762	2.23 (0.88 – 5.67)	0.090
Coverage with clothing (whole body)	17.5 (4.20 – 72.98)	<0.001	19.64 (2.52 – 152.68)	0.004
Use of sun screen (yes)	0.96 (0.37 – 2.47)	0.939	1.65 (0.42 – 6.48)	0.474

Nature of work (indoors)	33.63 (11.17 – 101.23)	<0.001	132.0 (10.9 – 1600.7)	<0.001
Time of sun exposure (at noon)	0.11 (0.06 - 0.20)	<0.001	0.13 (0.06 - 0.32)	<0.001

Note: **P*-value adjusted for Age, BMI at visit 1, Low-intense physical activity, Whole body cloth coverage, Job Status, Residential Area, Education, total-cholesterol-HDL ratio, Calcium intake (mg/day), Vitamin D intake (IU/day).
P-value<0.05 considered significant.

Table 4.3: The genotype distribution of *FokI* VDR gene polymorphisms among participants

***FokI* Genotype**	**All**	**Deficient (<50 nmol/L)**	**Non Deficient (>50 nmol/L)**	**OR (95 % CI)**	***P*-Value**	**Adj. OR (95%CI)**	**Adjusted *P*-value***
FF	204(59.1)	164 (60.1)	40 (55.6)	Reference			
Ff	117 (33.9)	91 (33.3)	26 (36.1)	0.85 (0.50 – 1.49)	0.577	0.44 (0.20 – 0.94)	0.035
ff	24 (7.0)	18 (6.6)	6 (8.3)	0.73 (0.27 – 1.96)	0.535	0.36 (0.08 – 1.52)	0.163
FF	204(59.1)	164 (60.1)	40 (55.6)	Reference			
Ff + ff	141 (40.9)	109 (39.9)	32 (44.4)	0.83 (0.49 – 1.40)	0.488	0.42 (0.20 - 0.88)	0.022
F	525 (76.1)	419 (76.7)	106 (73.6)	Reference			
f	165 (23.9)	127 (23.3)	38 (26.4)	0.85 (0.56 – 1.29)	0.434	0.51 (0.29 – 0.91)	0.021

Note: **P*-value adjusted for Age, BMI, low-intense physical activity, whole body cloth coverage, Job Status, residential Area, and education

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CHAPTER 5

MANUSCRIPT 2: *FOKI* VITAMIN D RECEPTOR GENE POLYMORPHISMS, GLUCOSE INTOLERANCE AND GESTATIONAL DIABETES RISK IN SAUDI WOMEN

5.1 Abstract

- **Background:** Gestational diabetes mellitus (GDM) is one of the major complications of pregnancy. GDM is common in pregnancies around the world (1% to 14%), with alarmingly high rates among Saudi women (39%). The reported prevalence of vitamin D deficiency is also higher in pregnant Saudi women (85%). Previous studies have linked vitamin D deficiency in pregnancy with a 1.6-times increased risk of developing GDM. The presence of the mutant *f* allele of the *FokI* vitamin D receptor (VDR) polymorphism has been shown to reduce vitamin D responsiveness. We expect that carriers of the *ff* genotype have a greater risk of GDM.
- **Aim:** This study examined whether there was a difference in GDM risk associated with the various genotypes of the *FokI* VDR polymorphism in pregnant Saudi women during their second trimester.
- **Materials and Methods:** A cross-sectional study of 368 pregnant Saudi women, including 108 GDM patients and 260 healthy subjects, was conducted in the antenatal clinics of three hospitals in Riyadh, Saudi Arabia (King Khaled University Hospital [KKUH], King Salman bin Abdulaziz Hospital, and King Fahad Medical City [KFMC]) between December 2013 and January 2016. The oral glucose tolerance test (OGTT) was administered at 24–28 weeks' gestation. We also measured fasting

blood glucose, insulin, and HbA_{1C}. The homeostasis models of insulin resistance and beta cell function were calculated using the HOMA-IR and HOMA-β, respectively. The *FokI* genotype of each woman was determined through allele-specific polymerase chain reaction (PCR).

- **Results:** About 29% of the study sample were diagnosed with GDM. The GDM patients were older, heavier, and had higher pre-pregnancy BMIs than the healthy subjects. As expected, the fasting blood glucose (FBG), fasting insulin, HbA_{1C}, HOMA-IR, and HOMA-β levels were also higher in the GDM patients. Among the GDM patients, the frequencies of the genotypes FF, F*f*, *ff*, and combined F*f*+*ff* were 60.2%, 33.3%, 6.5%, and 39.8%, respectively; among the healthy subjects, the frequencies of these genotypes were 61.1%, 31.5%, 6.9%, and 38.4%, respectively. While we could not determine a significant association between the *FokI* VDR polymorphism and GDM risk, we found that serum 25-hydroxyvitamin D was significantly and inversely associated with fasting blood glucose and HbA_{1C} within the group of patients carrying the *ff* minor allele genotype (r=-0.49, *P*=0.014 and r=-0.45, *P*= 0.026, respectively). Vitamin D deficiency was highly prevalent among the study sample, reaching up to 85.3%. This condition was also significantly associated with GDM (*P*=0.016) and glucose indices, such as fasting insulin, HOMA-IR and HOMA-β.
- **Conclusion:** Our study suggests that, within the Saudi population, the glucose-lowering effects of a higher vitamin D level may be dependent upon the *FokI* genotype.

5.2 Introduction

Gestational Diabetes Mellitus (GDM), defined as "carbohydrate intolerance of variable severity, with onset or first recognition during pregnancy",[1] is one of the more common complications of pregnancy. The global prevalence of GDM, which is from 1% to 14% of all pregnancies, depends on the population studied and the GDM diagnostic tests applied.[2] GDM can cause serious maternal and fetal morbidity and mortality. Short-term maternal complications of GDM include increased risk of maternal hypertension, pre-eclampsia, infection, cesarean delivery and premature birth, and in newborns hypoglycemia, macrosomia, shoulder dystocia or birth injury, increased intensive neonatal care, hyperbilirubinemia, respiratory distress syndrome, and polycythemia.[3,4] Long-term adverse maternal health outcomes of GDM include a 70% increase in the risk of developing Type 2 Diabetes (T2DM) within 10 years after delivery[5], and a 35 to 80% increased risk of developing GDM in a future pregnancy.[6] In addition, offspring of mothers with GDM are at increased risk of developing obesity, T2DM and cardiovascular disease during early childhood.[7] The pathogenesis of GDM remains unclear, [8] but likely is attributed to both genetic and lifestyle factors.

The role of vitamin D deficiency as a potentially modifiable nutritional risk factor for GDM has been highlighted in the literature. For example, a recent meta-analysis found that reduced vitamin D status was associated with a 45% increase in GDM risk. [8] Further support for a role of vitamin D status in blood glucose control in women with GDM has been supplied by two vitamin D supplementation studies.[9,10] One recent

randomized, double-blind placebo-controlled clinical trial found that a very high-dose of vitamin D supplementation (50,000 IU) every 2 weeks significantly reduced levels of insulin resistance in pregnant Chinese women with GDM .[9] Similarly, another randomized placebo-controlled trial found a significant reduction in fasting plasma glucose, serum insulin levels and HOMA insulin resistance in 56 Iranian women with GDM compared with the placebo group after supplementing them with calcium (1,000 mg/d) and vitamin D (50,000 IU/d) twice during the study period.[10]

In spite of bountiful sunlight in Saudi Arabia, vitamin D deficiency is common and reported to affect 72% of women of childbearing age.[11] A cross-sectional study found that out of 160 pregnant Saudi women, 90% of the study sample were either vitamin D deficient (25(OH)D ≤50 nmol/L) or vitamin D insufficient (25(OH)D = 50–74 nmol/L). [12] Similarly, another cross-sectional study of 1000 pregnant Saudi women reported that 86% were vitamin D deficient (mean: 30.5 ± 19.6 nmol/L).[13] This alarmingly high rate of vitamin D inadequacy needs to be carefully evaluated because many studies have linked vitamin D deficiency with adverse metabolic outcomes for both mother and their newborns. [14,15,16]

In addition, the prevalence of GDM among Saudi women is also exceedingly high, ranging between 10% to 51% depending on the diagnostic tool.[15] Important risk factors for GDM in Saudi women that have been identified include obesity, and the tradition of Saudi women to become pregnant at an older age.[16] Other commonly observed risk factors for GDM include: a family history of diabetes, gestational weight gain, history of GDM in previous pregnancies, a history of delivering a large baby (macrosomia).[15,16]

The suggested physiological mechanism behind the association of vitamin D status and risk of GDM is that the vitamin D receptor (VDR) is present in almost every tissue of the human body, including β-cells of the pancreas.[9] The active hormonal metabolite of vitamin D (1,25-dihydroxyvitamin D) binds to the VDR of pancreatic β cells and promotes insulin secretion and thereby may influence glucose homeostasis.[9, 17] Activation of the vitamin D receptor by its ligand can be modulated by many cellular factors, including single nucleotide polymorphisms (SNP) in the VDR gene itself, such as the *ff FokI* SNP (rs 2228570); previously reported as (rs10735810).[18] This VDR polymorphism is of particular interest because in vitro studies indicate that the *f* allele results in a nucleotide change in the gene sequence that results in the formation of a second upstream start site that results in the expression of a VDR protein that is 3 amino acids longer than the VDR coded by the *F FokI* VDR allele.[19] The longer VDR has been shown to be less responsive to 1,25-dihydroxyvitamin D than the shorter *F* allele version in activating target gene expression.[20] Thus, we hypothesized that Saudi mothers with poorer vitamin D status plus expression of the longer, less effective *f* version of the VDR protein would be at greatest risk of any adverse effects of vitamin D deficiency on glucose intolerance and the risk of GDM.

To our knowledge, only a few studies have investigated the association between the *FokI* VDR polymorphisms and GDM in Iran,[21] Saudi Arabia,[22,23] and most recently in Brazil,[23] but the findings were inconsistent and inconclusive due to the variability of the population and ethnic groups studied. A significant association was found between mutant *ff FokI* genotype carriers and the risk of GDM when compared with healthy subjects in Iranian and Brazilian population.[21,24] Nevertheless, El-Beshbishy et al. and Al-

Ghamdi failed to observe such an association in Saudi pregnant women.[22,23]

The current study was designed to determine to what extent the *f* allele of the *FokI* VDR gene polymorphism is a genetic biomarker associated with increased risk of GDM in Saudi women. We reasoned that the ability of vitamin D status to modulate glucose metabolism would be impaired in women with the *ff FokI* genotype. Our working hypothesis was that Saudi pregnant women carrying the (*f*) minor allele *FokI* VDR genotype will have an increased signs of glucose intolerance and a greater risk of developing GDM.

5.3 Methods

5.3.1 Study Design and Sample Population

This study is a part of a large prospective cohort study called "*Vitamin D and Pregnancy in Saudi Women*". Our cross-sectional study is a subset of these study participants and consisted of 368 pregnant Saudi women including 108 GDM patients and 260 healthy pregnant women who visited an antenatal clinic in their second trimester (24-28 weeks of gestation) at one of three different hospitals; King Khaled University Hospital (KKUH), King Salman bin Abdulaziz Hospital and King Fahad Medical City (KFMC) in Riyadh, the capital city of Saudi Arabia, between December 2013 and January 2016. The study has full ethical approval from the three hospitals to collect samples and patients data and also an approval was obtained from the Ethics Committee of the College of Science, King Saud University in Riyadh **(Appendix A).** Written informed consent was obtained from each patient **(Appendix B).**

5.3.1.1 Inclusion and Exclusion Criteria

Healthy pregnant Saudi women aged 18 to 40 years with no previous history of diabetes mellitus (type I or II) were enrolled in the study prior to 16 weeks of gestation. Subject exclusion criteria included: non-Saudi subjects; gestational age of over 16 weeks; women were excluded from the study if they were taking vitamin D supplements during pregnancy; unwillingness to deliver at the three hospitals; taking oral glucocorticoids; using drugs known to interfere with vitamin D or calcium absorption or parathyroid disorders; using any cardiac medication or diuretics; suffering from chronic hypertension or malabsorption syndrome; having chronic medical conditions or preexisting liver, kidney, calcium, and /or parathyroid conditions; or serious chronic disease conditions (epilepsy, cancer, other malignancy).

5.3.1.2 Recruitment and Medical Screening

Recruitment banners and brochures were placed in prenatal clinics in all three of the hospitals. Obstetricians were asked to introduce the research to their pregnant patients at their first prenatal appointment. Patients who met the criteria and consented to contribute in the present study were given the appropriate information. At their early pregnancy visit, prospective candidates were asked to sign consent forms that included information about their participation in the study, such as answering a questionnaire about demographic data collection, procurement of blood samples for biomarker measurement and DNA for genetic analyses, and anthropometric measurements. Permission for data collection from their medical records and stored blood stocks ifrom a bio-bank were also obtained **(Appendix A)**. The participants were also informed of their

right to withdraw from the study at any point, without it affecting their usual medical care.

5.3.2 Anthropometric Measurements

Anthropometric measurements were taken from the participants between 24 and 28 weeks gestation. These measures included weight (kg) and height (cm), used for calculating BMI (kg/m^2); reported pre-pregnancy weight (kg) and pre-pregnancy BMI (kg/m^2). Body weight, without shoes and wearing lightweight clothing, was measured to the nearest 0.1 kg (Digital Pearson Scale, ADAM Equipment Inc., USA). Height, to the nearest 0.5 cm, was measured at the early pregnancy visit only using the Digital Pearson Scale, while standing upright without shoes. Pre-pregnancy BMI was calculated from pre-pregnancy body weight recall and measured height.

5.3.3 Interview Questionnaire

At the second trimester visit (24-28 weeks of gestation), clinical data was collected. Pregnant women were questioned about GDM risk factors, such as parity (nulliparous or ≥ 1 multiparous), pre-pregnancy weight, previous miscarriage (yes/no), previous history of GDM, and family history of diabetes or any other family medical history. Participants were also asked about pregnancy-related symptoms, such as nausea, vomiting, morning sickness, frequent urination, headaches, abdominal bloating, constipation, acute respiratory or gastrointestinal viral or bacterial infections, any hospitalisation or medication prescribed, and the presence of any pregnancy-related complications.

5.3.4 Biochemical Assessment

During the antenatal visit between 24 and 28 weeks of gestation, participants were asked to fast > 10 hours for blood withdrawal. Blood samples (10 ml) were collected using sterile vacutainer blood collection apparatus. Whole blood, serum, and ethylene diamine tetra-acetic acid (EDTA) plasma were collected from the participants. All samples were aliquoted and stored in a -80ºC freezer for subsequent analyses. All the lab tests were performed on serum, except hemoglobin A_{1C} (HbA_{1C}), which was performed on whole blood. The blood samples were stored and analyzed at the *King Saud University Biomarkers Research Program* (BRP) Laboratory. It should be noted that the Biomarker Research Program (BRP) Laboratory is a participating entity in the vitamin D External Quality Assessment Scheme (DEQAS), and Quality Assurance (QA) standards are maintained by ISO 9000 and 17025. The QA department audits the BRP Laboratory at regular intervals.

5.3.4.1 Primary Outcome Measures

Our main outcome is the metabolic impact of the mutant *ff FokI* VDR genotype versus the wild type *FF FokI* VDR genotype on glucose intolerance and risk of GDM, measured by oral glucose tolerance test (OGTT) administered at 24-28 weeks gestation. For the OGTT, women were asked to fast for at least 10 hours, and a fasting blood sample was obtained and then a 75 g glucose drink was consumed (within a period of five minutes). Further blood collections took place at the 60- and 120-minute time points to assess glucose tolerance. Serum glucose (PG) was measured by using routine laboratory analysis (Konelab, Finland). The *International Association of Diabetes and Pregnancy Study Groups* (IADPSG) guidelines were applied to diagnose GDM based on the (fasting

≥5.1 mmol/l and or 1 h post glucose ≥10mmol/l h and/or 2 h post glucose load ≥8.5mmol/l) .[25]

5.3.4.2 DNA Extraction and Quantification

Genomic DNA was extracted from whole blood using innuPREP blood ini kits (Analytik Jena, Germany) following the manufacturer's instructions. Briefly, 200µl of whole blood sample was added to a 1.5 ml reaction tube containing lysis buffer and Proteinase K. The content was mixed by vortex and incubated at 60^0C for 10 minutes. The required amount of binding solution was added to the tube and transferred to a spin filter column with receiver tube and centrifuged for 1 minute at 12000 rpm. Washing of the column-bound DNA was performed using the kit's washing solution C followed by washing solution BS. In a final step, the spin filter was placed into a 1.5ml elution tube and 200µl of pre-warmed (at 60^0C) elution buffer was added. After centrifugation, the collected DNA was stored at -20◦C for further analysis. DNA concentration and purity (260/280) was determined using a Nano-Drop spectrophotometer.

5.3.4.3 *FokI* SNP Genotyping

The *Fok-I* SNP is in the VDR coding region (rs 2228570); previously reported as (rs10735810) and can be evaluated by allelic discrimination real-time PCR using pre-designed TaqMan genotyping assay from Applied Biosystems, Foster City, CA, USA (assay ID: C_12060045_20). Amplification reactions was performed in a volume of 10 µL containing 1X TaqMan genotyping Master Mix (Applied Biosystems), 1X mix of unlabeled PCR primers and TaqMan MGB probes, and 30 ng of template DNA. All amplification and detection was conducted in 96-well PCR plates using a Bio-Rad

CFX96 Real-Time PCR Detection System (Bio-Rad, Milan, Italy). Thermal cycling was initiated with a denaturation step of 10 min at 95 °C, followed by 45 cycles of 15 s at 95 °C and 90 s at 60 °C. After PCR was completed, allelic discrimination was analyzed using the Bio-Rad CFX Manager Software (Version 1.6, Bio-Rad). Genotype assignment was determined by plotting the end point relative fluorescent units (RFU) for one fluorophore (allele 1 on the x-axis) against the RFU for the other fluorophore (allele 2 on the y-axis) on the allelic discrimination. All PCR reactions were set up in a dedicated PCR area with dedicated PCR pipettes and reagents.

5.3.4.4 Other Measures

From the whole blood, HbA_{1C} was measured using a point-of-care device (Accu-Check Active, Roche Diagnostics, Mannheim, Germany). Fasting serum insulin was measured by COBAS e 411 Analyzer (Roche Diagnostics). The homeostasis model of insulin resistance (HOMA-IR) and homeostasis model of beta cell function (HOMA-β), were calculated to evaluate insulin resistance and basal pancreatic β-cell function using the following equations:

(HOMA-IR) = fasting insulin (μU/ml) × fasting FPG (mmol/l)/22.5 [14]

(HOMA-β) = 20 × fasting insulin(μU/ml)/[FPG(mmol/l) − 3.5] [14]

Higher HOMA-IR values indicated greater insulin resistance, while lower HOMA-β values indicated greater beta-cell dysfunction, as validated against gold standards.[26]

We also measured serum 25-hydroxyvitamin D (25(OH)D), which is the most reliable indicator of vitamin D status [27] through an electro-chemiluminescence binding assay 2012 (ECLIA) (Roche Diagnostics GmbA, Mannheim, Germany) and commercially available IDS kits (IDS Ltd, Boldon Colliery, Tyne & Wear, UK). The

inter- and intra-assay coefficients of variation (CV) for the 25(OH)D ELISA is 5.3% and 4.6%, respectively, with 100% cross-reactivity to 25(OH)D3 and 75% cross-reactivity to 25(OH)D_2. According to US Endocrine Society guidelines, the cutoff values for vitamin D deficiency is defined as serum 25(OH)D less than 50nmol/L. [27]

5.3.5 Statistical Analysis

The power analysis for this study is based on results from Aslani et al.[21] Using the G*Power Calculator for sample size effect size=0.22 α=0.05, power (1-β) = 0.95 and degrees of freedom=2, the total estimated sample size=321. Data were analyzed using SPSS statistical software (version 21.0, IBM). The normality of all quantitative variables was tested before performing the analysis, using Shapiro-Wilk Test. Quantitative normally distributed variables were presented as mean ± standard deviation (SD), while quantitative non-normally distributed variables were presented in median (25^{th} and 75^{th}) percentiles. Categorical data were presented as frequencies and percentages (%). Pearson's chi-square test was used to determine the differences between categorical variables. Comparisons between groups were performed using the independent sample t-test and the Mann-Whitney U test for normal and non-normal variables, respectively. The Pearson's and Spearman's rank correlation coefficients were determined to assess the linear relationship between quantitative variables for normally and non-normally distributed variables, respectively. Unadjusted and adjusted odds ratios were determined using logistic regression analysis to identify the risk factors associated with categorical outcome variables. Generalized multivariate analysis was also done to compare mean differences adjusted for age, BMI. A *P*-value of < 0.05 and 95% CI was finally applied to report statistical significance and the precision of the estimates.

5.4 Results

5.4.1 Demographic and Biochemical Characteristics of GDM vs. Normal Subjects

Table 5.1 presents the anthropometric and biochemical characteristics of the GDM and non-GDM groups of pregnant women. The prevalence of GDM was 29.3% (n=108/368). There was a significant difference in age, current BMI, and pre-pregnancy BMI between the GDM and normal subjects. GDM patients were significantly older and heavier than normal pregnant women ($P< 0.01$ and <0.01, respectively). Moreover, GDM patients were more obese (53.4%) compared to healthy pregnant women (30.2%), ($P<0.001$) **(Figure 5.1).** Compared with non-GDM group, fasting blood glucose (FBG), 1 h and 2 h OGTT, HbA_{1C}, fasting insulin, HOMA_IR, and HOMA-β in the GDM subjects were significantly increased. These significant increases remained even after adjusting for age and BMI. Although, medium serum 25(OH)D was low, but not different in the GDM and normal subjects (33.9 vs. 32.9 nmol/L, respectively $P = 0.686$), vitamin D deficiency however, was significantly higher in GDM group (93.2%) compared to normal subjects (82.2%) ($P =0.013$) **(Figure 5.1).**

Interestingly**,** there were no significant differences between GDM and normal subjects in regards to parity, family history of diabetes, and previous history of miscarriage. However, previous history of GDM was significantly higher in GDM than non-GDM groups **(**25.7% vs. 3.1%, respectively $P < 0.01$) **(Figure 5.1)**.

5.4.2 Risk Factors for GDM in Pregnancy

We further conducted a multivariate logistic regression analysis to assess the risk factors for GDM while adjusting for confounding factors (**Table 5.2**). Significant

independent variables that increased the risk of GDM included previous history of GDM (OR 9.25, 95% CI 3.14-27.22, $P < 0.001$), fasting insulin (OR 1.07, 95% CI 1.03-1.11, $P < 0.001$), HOMA_IR (OR 1.07, 95% CI 1.03-1.11, $P < 0.001$), obesity (OR 2.08, 95% CI 1.09-3.95, $P = 0.026$) and vitamin D deficiency (OR 2.96, 95% CI 1.22-7.19, $P = 0.016$).

Although HbA_{1C} was significantly higher in GDM than non-GDM group, it did not appear to be a risk factor for GDM. In fact, it lost its significance after adjusting for confounding factors (OR 1.50, 95% CI 0.74-3.02, $P = 0.263$).

5.4.3 *FokI* VDR Polymorphism and GDM

As shown in **Table 5.3**, the difference in the occurrence of the various *FokI* VDR genotypes in GDM and non-GDM participants was not significant. The *FokI* VDR genotypes are normally expressed as homozygous genotype "*FF*," heterozygous genotype "*Ff*," and mutant homozygous genotype "*ff*." The frequencies of *FF, Ff, ff,* and combined *Ff+ff* genotypes were 61.1% (225), 32.1% (118), 6.8% (25), and 39.8% (43) respectively, in the study population. Taking the *FF* genotype as a reference, the odds ratio for the risk of having GDM was not significant among the different *FokI* VDR genotypes, even after adjusting for important covariates such as age, BMI, family history of diabetes, obesity, previous history of GDM, fasting serum insulin, HbA_{1C} and vitamin D deficiency. The frequencies of genotypes *FF, Ff, ff,* and combined *Ff+ff* in women with GDM were 60.2%, 33.3%, 6.5%, and 39.8% respectively, while in the control group were 61.1%, 31.5%, 6.9%, and 38.4% respectively. The prevalence of *F* and *f* alleles for the *FokI* VDR polymorphisms in the two groups was also not significant (allele *F* vs. *f*; $P=0.495$), even after adjustments of covariates.

5.4.4 Biochemical Characteristics of the Subjects Depending on *FokI* VDR Genotypes and Alleles

Table 5.4 shows that women carrying the *f* allele had higher OGTT levels at 2 hours than subjects carrying the *F* allele (*P*= 0.017). Similarly, OGTT levels at 2 hours were significantly higher in combined *FokI* genotype *Ff+ff* than the *FF* genotype carriers (*P*= 0.019) (data not shown). Yet, we did not detect any significant association between other different biochemical variables and *FokI* VDR genotypes in all subjects.

5.4.5 Vitamin D Status and Glucose Indices

Table 5.5 presents the clinical and biochemical characteristics of vitamin D deficient versus non-deficient subjects. Vitamin D deficient subjects had significantly higher fasting insulin, HOMA_IR and HOMA1-β (*P*= 0.024, 0.007, 0.041, respectively).

5.4.6 Correlation Between Vitamin D and Other Parameters in Genotypes

Serum 25(OH)D was significantly and inversely associated with fasting blood glucose (FBG) (r= -0.49, *P*=0.014) **(Figure 5.2)** and HbA_{1C} (r = - 0.45, *P*=0.026) **(Figure 5.3)** in women carrying the *ff FokI* genotype. Moreover, there was a significant and positive association between serum 25(OH)D and HOMA1-β (r = 0.20, *P*<0.05) in patients carrying the *f* allele.

5.5 Discussion

Vitamin D deficiency was evident in 85.3% of our study sample. Despite the abundance of sunlight in Saudi Arabia, many previous studies have similarly reported a high prevalence of vitamin D deficiency among the Saudi population, with a higher rate

observed in younger females, especially in pregnant women and their offspring.[12,13] In line with our finding, both Al-Sheikh et al.[13] and Foda et al.[28] found that 86% of pregnant Saudi women, were vitamin D deficient. Several factors related to Saudi customs may play a role in vitamin D status, such as limited outdoor activities due to extremely high temperatures during the daytime as well as the tradition of wearing dark clothing that covers the whole body, thus preventing sun exposure and blocking the cutaneous production of vitamin D. Low consumption of foods containing vitamin D may be another important risk factor for vitamin D deficiency in Saudi Arabia.[12,13]

Poor vitamin D status is a known risk factor for glucose intolerance and the development of T2DM.[29] VDR gene polymorphisms are candidate single nucleotide polymorphisms associated with vulnerability to a variety of conditions and diseases, including glucose intolerance and T2DM.[18] Among the most common VDR polymorphisms studied, only the *FokI* VDR polymorphism has been shown to affect the VDR protein structure.[30] This polymorphism (denoted by *f*) results in a cytosine-to-thymine (C→T) nucleotide change in exon 2 of the coding region of the VDR gene, thus creating a second upstream start site due to a codon change from ACG to ATG.[19, 20] This start codon change causes the expression of a VDR protein that is 3 amino acids longer (427 versus 424 amino acids), which has been shown to have reduced transactivation ability.[19, 20] Therefore, we hypothesized that pregnant women with the *ff* genotype of the *FokI* polymorphism (all long VDR proteins) would be more likely to have impaired glucose tolerance than those with the *FF* genotype (all short VDR proteins). We also predicted that poor vitamin D status, in combination with the *ff* genotype, would be associated with a greater degree of glucose intolerance (determined via an OGTT at 24

weeks' gestation) and would be more likely to be diagnosed with GDM. However, our findings showed no significant associations between the *FokI* VDR polymorphism and the risk of GDM in pregnant Saudi women. In fact, the frequencies of *FokI* genotype and allele variants were almost identical in the wild-type (*FF)* carriers, *ff* homozygotes, and *Ff* heterozygotes among the GDM and control groups.

While previous studies have demonstrated the potential role of the *FokI* VDR polymorphism in the pathogenesis of GDM, they remain scarce and inconclusive. Our results agree with the following two studies conducted among the Saudi population.[22,23] In her small study, Al-Ghamdi did not observe any difference in the *FokI* VDR polymorphisms of 50 healthy, pregnant Saudi women and 50 GDM patients. [23] El-Beshbishy et al., using a sample size similar to our own, also did not detect any significant differences in the *FokI* VDR polymorphisms of 112 GDM patients and 218 healthy, pregnant Saudi women. [22]

In contrast, two studies found a significant association between the *FokI* VDR polymorphism and GDM. The first study was conducted by Aslani et al. on 303 pregnant Iranian women (142 GDM patients and 161 healthy subjects), which found that the *ff* genotype was more common in the GDM patients than in the healthy subjects (OR=1.783; 95% CI=0.774–4.107). [21] Interestingly, the healthy subjects had higher frequencies of the *F* allele (78.6% vs. 72.2%; $P<0.06$), suggesting that the *F* allele may contribute to decreasing the incidence of GDM.[21] The authors attributed the reason for this association to the dysfunction of the *f* VDR variant. As the mutant *f* allele is longer in structure and 40% less active than the *F* allele, carriers of the *f* allele are therefore predisposed to a higher risk of GDM.[21] The second study, which supports Aslani et al.'s

results, was recently conducted on a population from northeastern Brazil.[24] The authors examined whether the *FokI* VDR polymorphism was associated with GDM risk in pregnant women with a history of spontaneous preterm births (SPTBs).[24] The results revealed a significant association between the presence of the mutant *ff* genotype and GDM risk in SPTB patients, compared to patients who experienced normal deliveries (OR=4.71; CI 95%=1.1–22.30; *P*=0.044), as the frequency of the *ff* genotype was higher in the SPTB group (23% vs. 4.7%).[24] The authors therefore suggested that the *FokI* VDR polymorphism could be a biomarker for GDM.[24]

These previous findings should be interpreted with caution, however, as El-Beshbishy et al.[22] and Aslani et al.[21] measured fasting blood glucose alone to screen for GDM. The application of other criteria for the diagnosis of GDM, such as those of the International Association of Diabetes and Pregnancy Study Groups (IADPSG), results in an increased prevalence of GDM due to the inclusion of pregnant women with hyperglycemia.[25] Therefore, there is a possibility that the authors missed the high-risk patients, thus under-reporting the incidence of GDM, which may have affected the study outcomes. It was also not clearly indicated whether the previous investigators controlled for risk factors such as obesity, a family history of diabetes, or a history of microsomia, as only Aslani et al.[21] found the latter two factors to be significantly associated with GDM risk. Furthermore, the contradictory evidence of the previous studies could be explained by the small sample size, the heterogeneity of the analyzed samples, and the intra-individual variability in vitamin D status, which might have influenced the expression of VDR gene polymorphisms.[30] It should be noted that Aslani et al.[21] were the only investigators who measured the serum 25(OH)D levels; however, they did not study

the effect of vitamin D deficiency in combination with the genotype of the *FokI* VDR polymorphism on the patients' glucose intolerance or GDM risk.

Although our study did not result in a significant association between the *FokI* VDR polymorphism and the risk of GDM, we did find that carriers of the *f* allele had higher levels of blood glucose at two hours into the OGTT than carriers of the *F* allele. Moreover, in carriers of the *ff* genotype, serum 25(OH)D was found to be significantly and inversely associated with fasting blood glucose and HbA_{1C}. These observations might be explained by the fact that the VDR is expressed in many human tissues, including those involved in the regulation of glucose metabolism, such as pancreatic β cells.[8,19] The VDR is also known to alter glucose homeostasis via the insulin-like growth factor system.[8,12] Although the role of vitamin D and the VDR in regulating glucose homeostasis remains unclear, our results in the Saudi population suggest that the glucose-lowering effects of a higher vitamin D level may be dependent upon the *FokI* genotype.

It is well known that vitamin D may enhance insulin sensitivity by stimulating the expression of insulin receptors, thereby increasing insulin response to facilitate glucose transport.[31] In our results, we found evidence that supports the adverse effect of vitamin D deficiency on glucose tolerance and insulin sensitivity, as the levels of fasting insulin, and HOMA-IR were significantly higher in vitamin D-deficient subjects. These observations are congruent with other published studies reporting an association between lower serum 25(OH)D levels and higher HOMA-IR, which suggests that vitamin D deficiency during pregnancy may be related to insulin resistance and could therefore increase the risk of GDM.[32–35] Our findings are also in agreement with a recently published study that revealed a significant association between poor vitamin D status and

fasting blood glucose (P=0.002) or insulin sensitivity (P=0.001) in healthy Czech adults.[30]

In our study, although there were no observed, significant differences in vitamin D levels between the GDM and healthy subjects, even after adjusting for the cofounders of age and BMI, vitamin D deficiency appeared to be a risk factor for GDM. In fact, we found that the vitamin D-deficient subjects were 2.3 times more likely to develop GDM compared to their healthy counterparts (OR=2.29, 95%; CI=0.79–6.65; P=0.016). Our results are consistent with two previous studies that linked vitamin D deficiency with GDM[36,37] as well as a meta-analysis, which associated vitamin D deficiency in pregnancy with a 1.6-times increased risk of GDM.[38] However, in other studies, particularly in Saudi Arabia,[13] the Czech Republic,[39] Turkey,[32] India,[40] and Britain,[41] no associations were reported between vitamin D deficiency and the risk of GDM. Other factors that can potentially impact the results include geographical location, ethnicity, study design, sample size, lack of adjustment for confounding factors, heterogeneity of dietary factors, and differences in socio-economic status.[42,43]

GDM is considered a serious public health issue, as it has been associated with higher incidences of perinatal mortality and morbidity.[40] The prevalence of GDM in Saudi Arabia has risen steadily over the past two decades, from 12.5% in 2000[11] to 18.7% in 2013[44] and 24% in 2015.[45] These alarmingly large increments were measured using the 2013 criteria of the ADA and the WHO.[11,44,45] In our study, using the latest GDM diagnosis criteria of the IADPSG, we identified 108 participants (29.3%) with GDM. This rate appears to be high, and it corresponds well with the rising trend of GDM in Saudi Arabia; however, it should be noted that the IADPSG criteria includes pregnant

women with hyperglycemia as well as GDM patients, which may have affected the prevalence of GDM observed in our study population.[46,47] Three other studies conducted in Saudi Arabia used the same IADPSG criteria and, congruent with our results, found a high prevalence of GDM in their respective samples, amounting to 39.6%,[48] 36.6%,[49] and 39.4%.[16] These rates are extremely high when compared to the overall prevalence of GDM (17.8 %) found in the *Hyperglycemia and Adverse Pregnancy Outcome* (HAPO) cohort.[50] The HAPO was a large, multicenter, multinational, epidemiologic cohort study of 23,957 pregnant women at 24–32 weeks' gestation, who were similarly diagnosed using the IADPSG criteria.[4] According to the literature, GDM has genetic and environmental influences.[51] In addition to ethnic variation within a population, the most commonly reported risk factors for GDM in pregnant women include weight, parity, advanced maternal age, having delivered a macrosomic infant, having a previous history of GDM, and having a family history of diabetes.[49,54] Likewise, our study found that having a previous history of GDM, fasting insulin, HOMA-IR, obesity, and vitamin D deficiency (as discussed earlier) are strong, significant predicators of GDM. The association between having a previous history of GDM and the recurrence of GDM has been confirmed as well; as earlier studies found that GDM recurs in at least 30% of women with prior history.[53] Our findings are also supported by studies in different countries that found a significant association between pre-pregnancy obesity and GDM risk.[49,54]

The association between GDM and HbA_{1C} has been previously documented as well.[14] While we also observed a strong, significant relationship between GDM and HbA_{1C}, statistical significance was lost after adjusting for confounders.

We acknowledge that there are some limitations in our study, as cross-sectional studies are incapable of proving causation. In addition, our sample size was relatively small, especially in the group carrying the *ff* genotype of the *FokI* VDR polymorphism, thus making it difficult to assess the interaction between VDR gene polymorphisms and GDM risk. We also analyzed only one SNP of the VDR gene—albeit one that translates structurally diverse VDR proteins, which have different potentials in eliciting vitamin D-mediated gene expression. While there are other documented risk factors that might have masked the relationship between the *FokI* VDR polymorphism and GDM risk, such as gestational weight gain,[55] inflammation,[56] low levels of vitamin B12,[57] and low adiponectin,[58] we only controlled for the classical risk factors. Nevertheless, our study is one of the few in Saudi Arabia that assesses the association between the *FokI* VDR polymorphism and GDM while considering the confounding factors of age, BMI, parity, obesity, vitamin D deficiency, previous history of GDM, and family history of diabetes. We had strict standards for recruitment, as we excluded all patients taking vitamin D supplements. The accuracy of our vitamin D measurements is assured through our use of the ECLIA method, which is the gold standard in liquid chromatography–mass spectrometry (LC–MS/MS), and our results were included in the Vitamin D External Quality Assurance Scheme (DEQAS). We were also able to measure multiple outcomes related to glucose intolerance, such as insulin and HbA_{1C}, using the most accepted and cost-effective criteria worldwide (the IADPSG criteria).[59]

5.6 Conclusion

Contradictory results exist in the prevailing literature on the associations between VDR gene polymorphisms and GDM. While our study identified an effect on GDM risk

caused by vitamin D deficiency, we found no associations between the *ff* genotype of the *FokI* VDR polymorphism and the occurrence of GDM in the Saudi population. Regardless, our study is very encouraging, as it demonstrates that the glucose-lowering effects of a higher vitamin D level may be dependent upon the *FokI* genotype within the Saudi population. Our findings also confirm a high prevalence of GDM and vitamin D deficiency among pregnant Saudi women. A strong, significant association has been detected between vitamin D deficiency and GDM risk, along with other classical risk factors.

Potential interactions between vitamin D status and genetic polymorphisms in the pathogenesis of GDM are far from being fully comprehended. Nevertheless, given the role of vitamin D status in pregnancy outcomes and disease conditions other than GDM, it would be prudent to recommend increased vitamin D supplementation among this vulnerable population of women. Further studies are warranted, with sufficient statistical power, subjects of diverse ethnic backgrounds, and the inclusion of both classical and atypical risk factors for GDM as well as other genes in the vitamin D metabolic pathway, to confirm the potential association of VDR gene polymorphisms with GDM in different populations.

Table 5.1: Demographic and biochemical characteristics of GDM vs. normal subjects

Parameters	All	GDM	Non GDM	*P*-value	*P*-Value adjusted for Age and BMI
N	**368**	**108 (29.3)**	**260 (70.7)**		
Age (years)	29.1 ± 5.6	30.6 ± 6.0	28.4 ± 5.2	<0.001	
Parity	2.0 (1.0-4.0)	2.0(1.0-5.0)	2.0(1.0-3.0)	0.369	
Current-BMI (kg/m^2)	28.2 ± 6.1	30.3 ± 6.4	27.3 ± 5.9	<0.001	------
Pre-Pregnancy-BMI (kg/m^2)	26.9 ± 5.9	28.7 ± 5.9	25.9 ± 5.4	<0.001	------
FBG (mmol/L)	4.5 (4.1-5.0)	5.3 (4.8-5.6)	4.3 (3.9-5.6)	<0.001	<0.001
OGTT_1hrs (mmol/L)	7.4 (5.8-8.9)	10.1 (7.6-10.7)	6.7 (5.3-7.8)	<0.001	<0.001
OGTT_2hrs (mmol/L)	6.3 (5.3-7.7)	8.8 (6.6-10.2)	5.9 (5.1-6.9)	<0.001	<0.001
HbA_{1C} (%)	4.8 ± 0.5	5.0 ± 0.6	4.7 ± 0.5	<0.001	<0.001
Fasting Insulin (uU/ml)	7.5 (4.5-13.1)	9.7 (6.2-17.2)	6.5 (4.1-12.1)	<0.001	0.003
HOMA_IR	1.5 (0.9-2.5)	2.2 (1.3-3.9)	1.2 (0.7-2.2)	<0.001	<0.001
HOMA_β	141.9 (52.9-325.6)	371 (163.1-658.9)	100 (44.6-216.5)	<0.001	<0.001
25(OH)D (nmol/l)	33.4 (213-53.7)	33.9 (21.7-56.9)	32.9 (20.9-52.8)	0.772	0.686

Note: GDM: gestational diabetes mellitus, BMI: body mass index; FBG: fasting blood glucose; OGTT: Oral Glucose Tolerance Test; HbA_{1C}: haemoglobin A1c or glycated haemoglobin; HOMA_IR: homeostatic model assessment of insulin resistance; HOMA_β: homeostatic model assessment of beta cell function. Normally distributed variables are presented as Means ± SD. Non-normally distributed variables such as parity, FBG, OGTT 1,and 2 hrs, Fasting Insulin, HOMA_IR, HOMA_β, and vitamin D. are presented as Medians (25th and 75th percentile). *P*-value denotes significance at <0.05 and <0.01

Table 5.2: Predictors of GDM among pregnant women between 24-28 weeks gestation

Parameters	Univariate Analysis		Adjusted Model	
	OR (95% CI)	*P*-value	OR (95% CI) *	*P*-value*
Age (years)	1.05(1.00 - 1.10)	0.044	0.98(0.91 - 1.04)	0.448
Parity	1.11(0.96 - 1.30)	0.170	0.86 (0.68 – 1.08)	0.191
Current-BMI (kg/m^2)	1.05(1.02 - 1.10)	0.002	1.04(0.99 - 1.09)	0.168
Pre-Pregnancy-BMI (kg/m^2)	1.06(1.03 - 1.10)	0.001	1.05 (0.99 – 1.11)	0.072
Family history of Diabetes	1.13(0.63 - 2.00)	0.676	0.84(0.42 - 1.65)	0.607
Previous history of GDM	10.96 (4.14 – 29.0)	<0.001	9.25(3.14 - 27.22)	<0.001
History of Miscarriage	1.69(0.93 - 3.10)	0.087	1.36 (0.64 – 2.92)	0.426
HbA_{1C} (%)	2.44(1.51 - 3.92)	<0.001	1.50 (0.74 – 3.02)	0.263
Fasting Insulin (uU/ml)	1.07(1.03 - 1.10)	0.000	1.07 (1.03 – 1.11)	<0.001
HOMA_IR	56.8(12.66 - 254.60)	<0.001	75.34 (15.32 – 370.60)	<0.001
HOMA_β	0.48(0.24 - 0.96)	0.038	0.76 (0.36 – 1.57)	0.453
25(OH)D (nmol/l)	0.58(0.19 - 1.81)	0.352	1.00 (0.98 – 1.02)	0.966
Vitamin D deficiency (<50nmol/L)	2.96(1.22 – 7.19)	0.046	2.29(0.79 - 6.65)	0.016
Obesity	2.66(1.62– 4.36)	<0.001	2.08 (1.09 – 3.95)	0.026

Note: **P*-value adjusted for age, pre-pregnancy BMI, family history of diabetes, previous history of GDM, HbA_{1C} (%), and obesity.

Table 5.3: Comparison of the genotype and allele frequencies of the VDR *FokI* polymorphism in GDM and control groups

Parameters	**GDM**	**Non-GDM**	**Odd Ratio (95% CI)**	***P*-Value**	**Adjusted OR (95% CI)**	**Adjusted *P*-Value**
N	**N=108 (29.3%)**	**N=160 (70.7%)**				
FokI- rs10735810						
FF	65 (60.2)	160 (61.1)	1		1	
Ff	36 (33.3)	82 (31.5)	0.96 (0.38-2.40)	0.926	1.26 (0.48-3.31)	0.426
ff	7 (6.5)	18 (6.9)	1.08 (0.66-1.76)	0.755	1.28 (0.76-2.14)	0.289
Ff+ff	43 (39.8)	100 (38.4)	1.06 (0.66-1.68)	0.808	1.29 (0.78-2.07)	0.256
F	166 (76.9)	402 (77.3)	1		1	
f	50 (23.1)	118 (22.7)	1.03 (0.70-1.50)	0.704	1.51 (0.78-1.70)	0.368

Note: OR: Odds ratio (95% CI), P-value adjusted for Age, Pre-pregnancy BMI, family history of diabetes, obesity, previous history of GDM, fasting serum insulin, Hbg A_{1c} and vitamin D deficiency.

Table 5.4: Clinical and biochemical characteristics of the subjects depending on their alleles

Parameters	***F***	***f***	***P*-value**
N	**568**	**168**	
Age (years)	29.2 ± 5.9	28.3 ± 5.3	0.073
Parity	2.0 (1.0-4.0)	2.0(1.0-3.0)	0.755
Current-BMI (kg/m^2)	28.5 ± 6.5	27.6 ± 5.7	0.121
Pre-Pregnancy-BMI (kg/m^2)	27.2 ± 6.2	26.3 ± 5.2	0.080
FBG (mmol/L)	4.5 (4.1-4.9)	4.5 (4.1-4.9)	0.625
OGTT_1hrs (mmol/L)	7.3 (5.8-8.8)	8.0 (5.9-9.5)	0.122
OGTT_2hrs (mmol/L)	6.2 (5.2-7.7)	6.6 (5.8-7.8)	0.017
HbA_{1C} (%)	4.8 ± 0.5	4.9 ± 0.7	0.099
Fasting Insulin (uU/ml)	7.5 (4.4-12.9)	7.7 (5.6-15.6)	0.967
HOMA_IR	1.5 (0.9-2.6)	1.3 (0.9-2.8)	0.975
HOMA_β	141.7 (52.6-315.9)	136.9 (57.1-360.1)	0.772
25(OH)D (nmol/l)	33.6 (22.4-53.7)	31.6 (19.3-52.2)	0.303

Note: Data represented Mean ± SD and Median (1st and 3rd) percentile for Gaussian and non-Gaussian variables. *P*-Value denotes significance at <=0.05 and 0.01.

Table 5.5: Clinical characteristics of the vitamin D deficient and non-deficient pregnant Saudi women

Parameters	Vitamin D deficient 25(OH)D<50 nmol/l	Non- deficient 25(OH)D >50 nmol/l	*P*-value
N	**260 (85.3)**	**108 (14.7)**	
Age (years)	28.9 ± 5.7	29.4 ± 5.2	0.470
Parity	2.0 (1.0-4.0)	1.0 (1.0-3.0)	0.202
Current-BMI (kg/m^2)	28.2 ± 6.5	28.1 ± 5.6	0.835
Pre-Pregnancy-BMI (kg/m^2)	26.8 ± 6.1	26.9 ± 5.5	0.852
FBG (mmol/L)	4.5 (4.1-4.9)	4.2 (3.9-5.1)	0.081
OGTT_1hrs (mmol/L)	7.3 (5.8-8.7)	8.4 (6.7-9.5)	0.114
OGTT_2hrs (mmol/L)	6.3 (5.3-7.4)	6.8 (5.6-9.1)	0.756
HbA_{1C} (%)	4.8 ± 0.5	4.8 ± 0.6	0.712
Fasting Insulin (uU/ml)	7.6 (4.6-13.1)	6.3 (4.5-12.9)	**0.024**
HOMA_IR	1.5 (0.9-2.5)	1.3 (0.9-2.7)	**0.007**
HOMA_β	146.8 (59.3-319.9)	111.1 (56.8-348.3)	**0.041**
25(OH)D (nmol/l)	29.9 (20.1-46.7)	66.7 (43.4-85.3)	**<0.001**

Note: Data represented Mean ± SD and Median (25th and 75th) percentile for Gaussian and non-Gaussian variables. *P*-Value Denotes significance at *P* <0.05 and 0.01

Figure 5.1 Demographic and biochemical parameters in relation to GDM status

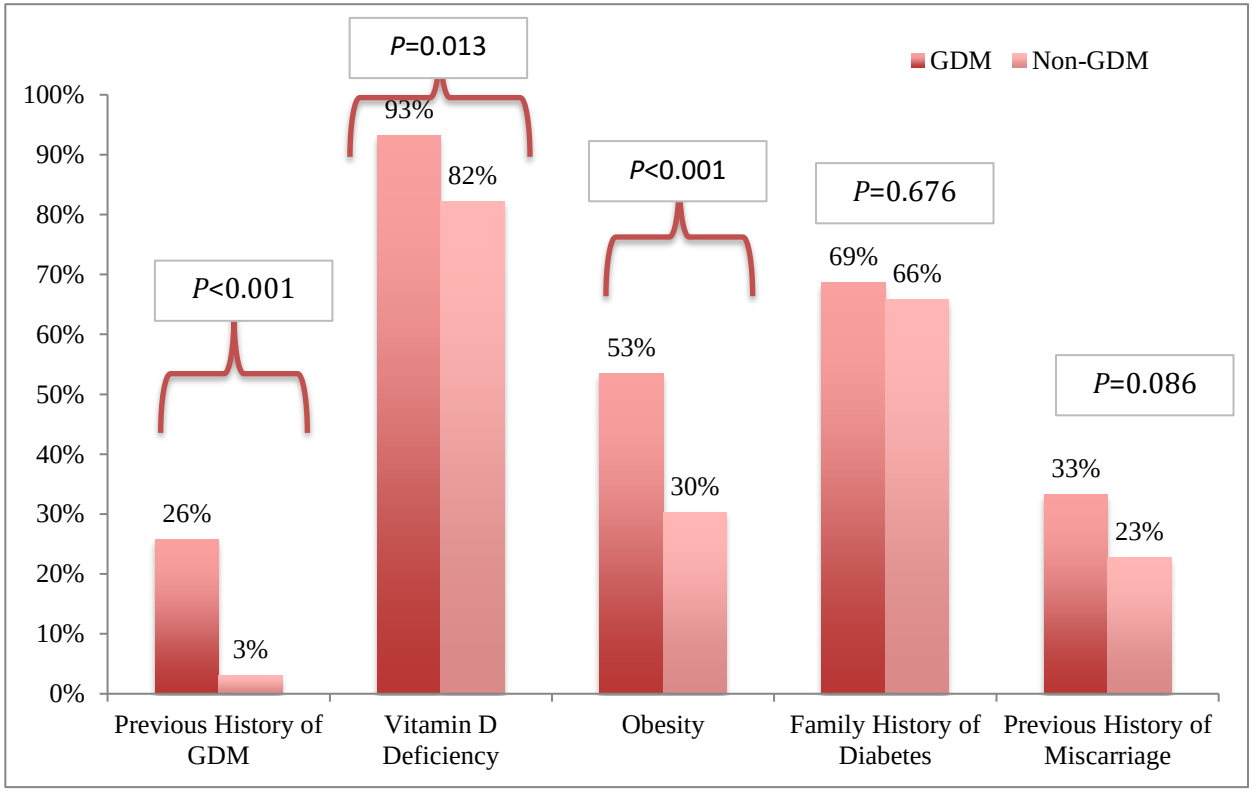
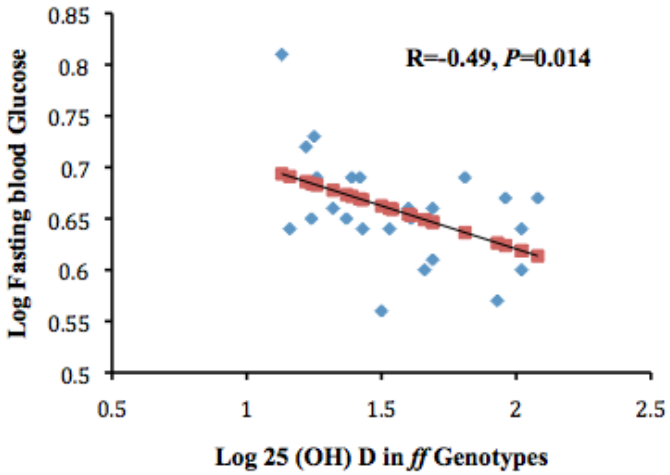
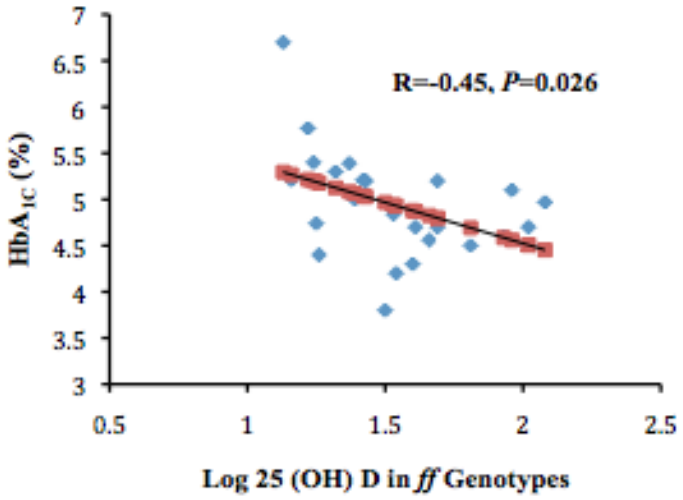


Figure 5.2 Correlations between Log vitamin D (nmol/L) in *ff FokI* genotypes versus (A) FBG, (B) HbA_{1C}

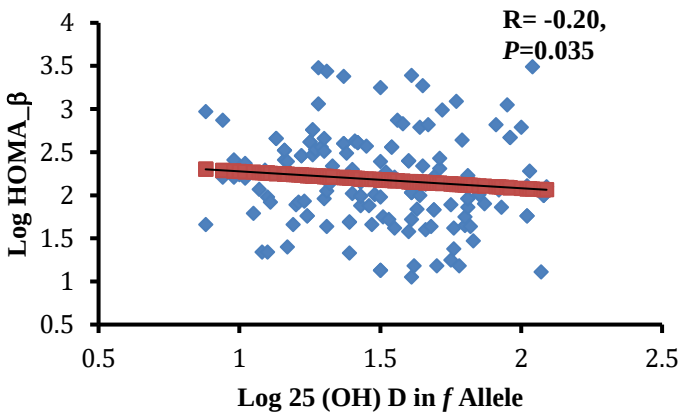


A



B

Figure 5.3 Correlation between Log vitamin D (nmol/L) in *f FokI* allele versus HOMA_β



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CHAPTER 6

MANUSCRIPT 3: VITAMIN D, VITAMIN D RECEPTOR POLYMORPHISM, AND METABOLIC SYNDROME IN PREGNANT SAUDI WOMEN

6.1 Abstract

- **Background:** Metabolic Syndrome (MS) is a serious health concern and its prevalence is rising significantly in Saudi Arabia. It is also more common in females than in males. Pregnant women with MS have a higher risk of developing gestational diabetes (GDM) and preeclampsia during pregnancy and a great increased risk of future chronic diseases such as cardiovascular disease (CVD) and Type 2 diabetes mellitus (T2DM). In addition, it has been previously reported that complications during pregnancy may contribute negatively to the health of the unborn infant from gestation to adulthood and cause an increased risk for morbidity and mortality. Previous studies have linked Vitamin D receptor (VDR) gene polymorphism to MS in adults. However, the association between VDR gene polymorphisms with maternal MS and its components in pregnancy has not yet been clarified.
- **Aim**: The objective of this study was to investigate the association of the FokI VDR genotype as a risk factor for MS and its components in pregnant Saudi women.
- **Materials and Method:** A cross-sectional study of 368 pregnant Saudi women (44 with MS and 324 without MS) who visited antenatal clinics of three hospitals in Riyadh, Saudi Arabia (King Khaled University Hospital [KKUH], King Salman bin Abdulaziz Hospital, and King Fahad Medical City [KFMC]) between December 2013 and January 2016. Fasting serum glucose, $HbgA_{1C}$, insulin, HDL-C, LDL-C, TG, TC,

25(OH) D and blood pressure were measured in the second trimester (at 24-28 weeks of pregnancy. The *Fokl* VDR genotype of each woman was determined by allele-specific polymerase chain reaction (PCR).

- **Results**: About 12% of the study population had MS. *FokI* VDR genotyping frequencies for *FF, Ff,* and *ff* genotypes were 62.7% (203), 31.5% (102), and 5.9% (19), respectively, in healthy pregnancies and 50.0% (22), 36.4% (16), and 13.6% (6), respectively, in patients with MS. The minor *FokI ff* genotype was a significant risk factor for MS (OR = 2.91; 95% CI, 1.05-8.1; *P*= 0.039).
- **Conclusion**: This observation suggests that the presence of the *ff FokI* VDR genotype is genetic marker of MS risk in pregnant Saudi women. However, we did not find a significant association between *FokI* VDR genotype and components of MS in these pregnant women.

6.2 Introduction

Metabolic syndrome (MS) is a cluster of abnormal metabolic health disorders that includes 3 or more of the following 5 characteristics: elevated blood pressure, central obesity, dyslipidemia, high cholesterol and elevated fasting blood sugar.[1] MS is a growing worldwide health problem and is a risk factor for developing cardiovascular disease (CVD) and type 2 diabetes mellitus (T2DM).[2] The prevalence of MS has been rising globally and increasing from about 20% to 40% in the adult population.[3] In the United States, for example, about 25% of the adult population suffers from MS,[4] whereas in Saudi Arabia, the prevalence of MS is much higher, ranging between 13.6 and 57%.[5] Differences in the prevalence estimates of MS in different geographic areas may by

affected by gender variations in the population studied, different targeted populations or age groups studied, as well as possible differences in the criteria used in its definition.[5] Moreover, a recent study from Saudi Arabia revealed that 43.42% of the total cohort of 12,126 study participants, aged 18 years and above, had two or more risk factor components for MS.[6]

The prevalence of MS in general is higher in females than in males.[4] Al-Nozha and colleagues, found a higher ratio of MS in Saudi females than in males, 42%, and 37.2%, respectively.[7] A systematic review conducted by Aljefree and Ahmed in the Gulf countries found a high prevalence of CVD and its associated risk factors, particularly obesity among women in this area.[8] Another large cohort study in Saudi Arabia; Riyadh *Mother and Baby Study (RAHMA*), found that more than 68% of the 14,568 pregnant women studied were either overweight or obese and 24% of the total cohort developed gestational diabetes. [9] The prevalence of these two risk factors for MS; namely, obesity and gestational diabetes were among the highest in the world.[9]

Identification of MS in pregnant women is important because they are at increased risk of developing maternal complications, such as preeclampsia and coma, as well as adverse perinatal effects, such as malformations, neonatal hypoglycemia, neonatal macrosomia, jaundice, and preterm birth.[1,4,10] Moreover, Rodie et al. reviewed different studies related to complications during pregnancy and their outcomes and reported that multiple complications, such as preeclampsia, preterm birth and intra-uterine growth restriction, carries a seven-fold additive risk of future diseases, including maternal CVD.[11] In addition, an increasing body of epidemiologic evidence indicates that offspring of high-risk pregnancies accompanied by complications and adverse birth outcomes are

at increased risk of developing MS, CVD, and T2DM later in life.[1,10], Gosadi has reported that "CVD is one of the leading causes of death in the adult Saudi population where the increase in cardiovascular-related mortality is augmented by the rise in the prevalence of MS."[12]

The alarmingly pandemic rate of MS in Saudi Arabia and elsewhere can be attributed to many etiological factors. For example, in Saudi Arabia, rapid urbanization, globalization, and improvements in socioeconomic status have resulted in a significant change in lifestyle.[13] A recent systematic review also found a very high prevalence of physical inactivity among Saudi women, ranging from 53.2% to 98.1%.[14] These authors attributed the high prevalence of sedentary lifestyle and obesity in Saudi women to socio-cultural factors like the banning of driving and physical education in girls' public schools.[14] The dietary pattern of the Saudi Arabian diet has also changed to a more Westernized diet with increased consumption of high-energy-dense foods and processed foods that are high in fat, sugar and salt.[13] As a result, non-communicable diseases (NCD) such as obesity, diabetes, hypertension and high cholesterol have emerged as a significant problem, and are affecting a growing number of people in Saudi Arabia.[13,15] Rahim et al. reported that in Arab countries there were more than one million deaths in the year of 2008 from NCD, which accounted for 60% of all deaths.[16] Although all these risk factors in addition to aging are considered to be primary etiological factors for developing MS, genetic predisposition could be another important factor in the prediction of risk for MS.[3]

The activity of 1,25-dihydroxyvitamin D, the active hormonal form of vitamin D, is mediated through the cellular vitamin D nuclear receptor (VDR) protein.[17] VDR is

expressed in most body tissues and has been shown to be important in calcium homeostasis and bone metabolism, as well as other non-calcemic actions.[18,19] Single nucleotide polymorphisms (SNPs) are common in the genome and can sometimes affect gene expression or function. The VDR gene has several recognized SNPs that could alter the functioning of the VDR.[20] Among these VDR polymorphisms, the *FokI* VDR gene polymorphism has been of interest because it occurs in the transcription start codon, and its polymorphic form (*f*) results in a change in the start codon position such that the expression of the VDR protein is three amino acids longer than the wild-type (*F*) allele.[17] The longer VDR protein has been shown to be less active than the shorter wild-type version. This shorter *FokI* VDR allelic variation has also been found associated with various adverse disease outcomes in some epidemiologic studies.[21,22] The association of *FokI* VDR genotypes with pregnancy complications has been studied, but has resulted in mixed findings.[23]

Vitamin D status is largely dependent on sunlight exposure and is reflected in higher serum 25-hydroxyvitamin (25(OH)D), a biomarker of vitamin D status. However, it is evident that vitamin D deficiency is quite prevalent among pregnant women in Saudi Arabia, despite the abundance of sunlight all year around.[24] Vitamin D deficiency itself has been linked to MS. Meta-analysis of cross-sectional studies found a significant inverse association between the level of serum 25(OH)D and the risk of MS.[25] In another systematic review, it was observed that the highest levels of serum 25(OH)D were associated with a 43% reduction in CVD, T2DM and MS.[18]

Several studies have examined the relationship between VDR gene polymorphisms and the risk for MS or its components in adults. These studies found that *FokI* VDR gene polymorphisms were associated with blood pressure, insulin resistance, BMI and lipid profile, but the findings from these studies in different ethnic groups remained inconclusive. [2,3,21,22, 26,27] To our knowledge, there have been no studies that have investigated the association between VDR gene polymorphisms and MS and its components among pregnant Saudi women. Given the endemic nature of low vitamin D status and vitamin D deficiency found in the Saudi population, this group may be particularly susceptible to vitamin D-related adverse events due to the presence of the relatively ineffective longer VDR protein found in women with the *ff* VDR genotype. Therefore, our study was designed to investigate the association of VDR *FokI* genotypes with the risk of MS and its individual components in pregnant Saudi women living in Riyadh, the capital city of Saudi Arabia.

6.3 Methods

6.3.1 Study Design and Sample Population

This study is a part of a large prospective cohort study called "*Vitamin D and Pregnancy in Saudi Women*". Our cross-sectional study is a subset of these study participants and consisted of 368 pregnant Saudi women, which included 324 healthy pregnant women and 44 pregnant women with MS, among subjects who visited an antenatal clinic in their second trimester (24-28 weeks of gestation) at one of three Saudi hospitals; King Khaled University Hospital (KKUH), King Salman bin Abdulaziz Hospital and King Fahad Medical City (KFMC) in Riyadh, the capital city of Saudi

Arabia, between December 2013 and January 2016. The study has full ethical approval from the three hospitals to collect samples and patient data and approval from the Ethics Committee of the College of Science, King Saud University in Riyadh. **(Appendix A)**. Written informed consent was obtained from each patient **(Appendix B)**.

6.3.1.1 Inclusion and Exclusion Criteria

Healthy pregnant Saudi women, aged 18 to 40 years, with no previous history of diabetes mellitus (type I or II) were enrolled in the study prior to 16 weeks of gestation. Subject exclusion criteria included: non-Saudi subjects; gestational age of over 16 weeks; if they were taking vitamin D supplements during pregnancy; unwillingness to deliver at any of the three hospitals; taking oral glucocorticoids; using drugs known to interfere with vitamin D or calcium absorption or parathyroid disorders; using any cardiac medication or diuretics; suffering from chronic hypertension or malabsorption syndrome; having chronic medical conditions or preexisting liver, kidney, calcium, and /or parathyroid conditions; or serious chronic disease conditions (epilepsy, cancer, other malignancy).

6.3.1.2 Recruitment and Medical Screening

Recruitment banners and brochures were placed in prenatal clinics in all three of the hospitals. Obstetricians were asked to introduce the research to their pregnant patients at their first prenatal appointment. Patients who met the criteria and consented to contribute in the present study were given the appropriate information. At their early pregnancy visit, prospective candidates were asked to sign consent forms that included information about their participation in the study, such as answering a questionnaire

about demographic data, procurement of blood samples for biomarker measurement and DNA for genetic analyses, and anthropometric measurements. Permission for data collection from their medical records and stored blood stocks from a bio-bank were also obtained **(Appendix A)**. The participants were also informed of their right to withdraw from the study at any point without it affecting their usual medical care.

6.3.2 Anthropometric Measurements

Anthropometric measurements were taken from the participants between 24 and 28 weeks gestation. These measures included weight (kg) and height (cm), used for calculating body mass index (BMI) (kg/m^2); reported pre-pregnancy weight (kg) and pre-pregnancy BMI (kg/m^2). Systolic and diastolic blood pressures (mmHg) were also recorded.

Body weight, without shoes and wearing lightweight clothing, was measured to the nearest 0.1 kg (Digital Pearson Scale, ADAM Equipment Inc., USA). Based on pre-pregnancy weight as self-reported during the prenatal visit, pregnant women were classified according to the WHO BMI definitions as follows: underweight: <18.5 kg/m^2; normal weight: 18.5–24.9 kg/m^2; overweight: 25.0 - 29.9 kg/m^2; or obese: ≥ 30.0 kg/m^2.[28]

Height, to the nearest 0.5 cm, was measured at the early pregnancy visit only using a stadiometer (Digital Pearson Scale), while standing upright without shoes. Pre-pregnancy BMI was calculated from pre-pregnancy body weight recall and measured height. Blood pressure (mm Hg) was measured using a mercury sphygmomanometer, while the patient was relaxed and seated. Per the criteria set out by the International

Society for the Study of Hypertension in Pregnancy, gestational HTN was identified as BP≥140/90 mm Hg.[29]

6.3.3 Biochemical Assessment

During the antenatal visit between 24 and 28 weeks of gestation, participants were asked to fast > 10 hours for blood withdrawal. Blood samples (10 ml) were collected using sterile vacutainer blood collection apparatus. Whole blood, serum, and ethylene diamine tetra-acetic acid (EDTA) plasma were collected from the participants. All samples were aliquoted and stored in a -80ºC freezer for subsequent analyses. All the lab tests were performed on serum, except hemoglobin A_{1C} (HbA_{1C}), which was performed on whole blood. The blood samples were stored and analyzed at the *King Saud University Biomarkers Research Program (BRP)* Laboratory. It should be noted that the Biomarker Research Program (BRP) Laboratory is a participating entity in the vitamin D External Quality Assessment Scheme (DEQAS), and Quality Assurance (QA) standards are maintained by ISO 9000 and 17025. The QA department audits the BRP Laboratory at regular intervals. Serum 25(OH)D was measured by electro-chemiluminescence binding assay 2012 (ECLIA) (Roche Diagnostics GmbA, Mannheim, Germany) and commercially available IDS kits (IDS Ltd, Boldon Colliery, Tyne & Wear, UK). The inter- and intra-assay coefficients of variation (CV) for 25(OH)D ELISA is 5.3% and 4.6%, respectively, with 100% cross-reactivity to 25(OH)D3 and 75% cross-reactivity to 25(OH)D2. According to US Endocrine Society guidelines, the cutoff values for vitamin D deficiency is defined as serum 25(OH)D less than 50 nmol/L, and for sufficiency serum 25(OH)D more than 75nmol/L. [30]

6.3.3.1 Primary Outcome Measures

Our main outcome measure was to study the metabolic impact of the *ff FokI* VDR genotype versus the *FF* wild type VDR genotype on the risk of MS. Participants were divided into two groups, cases with MS and controls without MS, according to the *International Diabetes Federation* (IDF) definition with some attention taken into account to accommodate to our study population of pregnant women.[4] In particular, for a pregnant women to be defined as having MS according to IDF definition, she must have 3 or more of the following risk factors: pre-pregnancy body mass index (BMI) > 30 kg/m^2; level of serum triglyceride(TG) ≥ 150 mg /dL; serum high-density lipoprotein (HDL-cholesterol level < 50 mg/dL; fasting serum glucose level ≥ 100 mg/dL, and blood pressure level ≥ 130/85 mm Hg. **[4]** Healthy pregnant control subjects were those who did not match the criteria employed for the selection of MS subjects.

6.3.3.2 Other Measures

After an overnight fast (>10 h), participants were requested to return to their prenatal clinics for blood withdrawal. A 6-mL sample of fasting venous blood was collected and transferred immediately to a non-heparinized tube for centrifugation. Serum was then transferred to a pre-labeled plain tube, stored in ice, and delivered to the Biomarker Research Center in King Saud University on the same day. Fasting serum samples were stored in a −20 °C freezer prior to analysis to facilitate availability for later analysis.

Fasting serum glucose (FBG) and lipid profile (including total cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) were all measured using a chemical analyzer (Konelab,

Vantaa, Finland). Dyslipidemia is defined by presence of one or more abnormal serum lipid biomarkers. The cut-off of dyslipidemia, according to IDF guidelines, was as follows: TC ≥ 5.2 mmol/L (200 mg/dl), LDL ≥ 3.4 mmol/L (130 mg/dl), HDL <1.3 mmol/L (50 mg/dl), or TG >1.7 mmol/L (150 mg/dl), or a combination thereof. [31]

From the whole blood, HbA_{1C} was measured using point-of-care (POC) devices (Accu-Check Active, Roche Diagnostics, Mannheim, Germany).

Fasting serum insulin was measured by COBAS e 411 Analyzer (Roche Diagnostics). The homeostasis model of insulin resistance (HOMA-IR) was calculated to evaluate insulin resistance using the following equations: (HOMA-IR) = fasting insulin (μU/ml) × fasting FPG (mmol/l)/22.5. Higher HOMA-IR values indicated greater insulin resistance.[31]

6.3.3.3 DNA Extraction and Quantification

Genomic DNA was extracted from whole blood using innuPREP blood mini kits (Analytik Jena, Germany) following the manufacturer's instructions. Briefly, 200µl of whole blood sample was added to a 1.5 ml reaction tube containing lysis buffer and Proteinase K. The content was mixed by vortex and incubated at 60 ^{0}C for 10 minutes. The required amount of binding solution was added to the tube and transferred to a spin filter column with receiver tube and centrifuged for 1 minute at 12000 rpm. Washing of the column-bound DNA was performed using the kit's washing solution C followed by washing solution BS. In a final step, the spin filter was placed into a 1.5ml elution tube and 200µl of pre-warmed at 60 ^{0}C elution buffer was added. After centrifugation, the collected DNA was stored at -20 ^{0}C for further analysis. DNA concentration and purity (260/280) was determined using a Nano-Drop spectrophotometer.

6.3.3.4 *FokI* SNP Genotyping

The *FokI* SNP is in the VDR coding region (rs 2228570); previously reported as (rs10735810) and can be evaluated by allelic discrimination real-time PCR using pre-designed TaqMan genotyping assay from Applied Biosystems, Foster City, CA, USA (assay ID: C_12060045_20). Amplification reactions was performed in a volume of 10 µL containing 1X TaqMan genotyping Master Mix (Applied Biosystems), 1X mix of unlabeled PCR primers and TaqMan MGB probes, and 30 ng of template DNA. All amplification and detection was conducted in 96-well PCR plates using a Bio-Rad CFX96 Real-Time PCR Detection System (Bio-Rad, Milan, Italy). Thermal cycling was initiated with a denaturation step of 10 min at 95 °C, followed by 45 cycles of 15 s at 95 °C and 90 s at 60 °C. After PCR was completed, allelic discrimination was analyzed using the Bio-Rad CFX Manager Software (Version 1.6, Bio-Rad). Genotype assignment was determined by plotting the end point relative fluorescent units (RFU) for one fluorophore (allele 1 on the x-axis) against the RFU for the other fluorophore (allele 2 on the y-axis) on the allelic discrimination. All PCR reactions were set up in a dedicated PCR area with dedicated PCR pipettes and reagents.

6.3.4 Statistical Analysis

Data were analyzed using SPSS (version 21.0, IBM). Continuous data are presented as mean ± standard deviation (SD) for Gaussian variables and non-Gaussian variables are presented as median (25^{th} and 75^{th}) percentiles. Categorical data are presented as frequencies and percentages (%). All continuous variables were checked for normality using Kolmogorov-Smirnov test. Non-Gaussian variables were log-

transformed prior to parametric analysis. Independent T-test, analysis of variance (ANOVA), Mann-Whitney and Kruskal-Walis H tests were used to compare mean differences in Gaussian and non-Gaussian variables. Odds-ratio and X^2 tests were done using binary logistics and multinomial logistic regression. A *p*- value <0.05 was considered statistically significant.

6.4 Results

6.4.1 General Characteristics and Components of MS

Table 6.1 presents the characteristics of the subjects with and without MS. In our study, the prevalence of MS was 12%. Pregnant women with MS were significantly older and have higher pre-pregnancy BMI than the controls (*P*< 0.02 and <0.01, respectively). The mean age of the 44 women with MS was 31 (±7) years, and the mean age of 324 controls was 29 (±5) years. Moreover, median serum 25(OH)D was lower in the MS subjects than controls (29.9 and 33.7 nmol/L, respectively), but did not reach statistical significance (*P*=0.186), as both groups had high prevalence of vitamin D deficiency (*P*=0.199). Compared with the controls, diastolic blood pressure, $HbgA_{1C}$, fasting serum glucose, HOMA_IR, insulin, and TG were significantly higher in pregnant women with MS, while HDL cholesterol was significantly lower (1.6±0.4 vs. 1.3±0.5 mg/dl, *P*<0.001). These findings remained significant even after adjusting for age and pre-pregnancy BMI.

6.4.2 *FokI* VDR Gene Polymorphisms and MS Risk

As shown in **Table 6.2**, the difference in the occurrence of the genotypes in *FokI* between individuals with MS and the control group was statistically significant even after

the adjustment for age. The *FokI* VDR genotypes are normally expressed as dominant homozygous genotype "*FF*", heterozygous genotype "*Ff*", and mutant homozygous genotype "*ff*". The frequencies of genotype *FF*, *Ff*, and *ff* in women with MS were 50%, 36%, and 14% respectively, while in the control group were 63%, 32%, and 6%, respectively, which suggests that the genotype *ff* is a risk factor for MS (OR = 4.17; 95% CI, 1.42-12.2, *P*=0.009) in pregnant Saudi women. The prevalence of *F* and *f* alleles for the *FokI* VDR polymorphisms in the two groups were statistically significant (allele *F* vs. *f*; *P*=0.017), which suggests that the *f* allele is a genetic risk factor for MS in this population.

6.4.3 Clinical Characteristics of the *FokI* VDR Gene Polymorphisms in MS and Control Group

The distribution of some clinical and biochemical variables according to *FokI* VDR genotype that were observed in MS participants and pregnant controls are shown in **Table 6.3**. The pregnant women without MS with the *FF* genotype had a higher pre-pregnancy and current BMI than pregnant women with either the F*f* or *ff* genotypes (*P*= 0.021, 0.027, respectively). We did not observe any association between VDR genotype and individual components of MS in pregnant women.

6.4.4 *FokI* VDR Gene Polymorphism x Components of MS

We conducted a multinomial logistic regression to better understand the relationship between different genotypes and alleles of *FokI* VDR polymorphisms and obesity, hypertension, low HDL, hypertriglyceridemia, hypercholesterolemia, dyslipidemia, and GDM **(Table 6.4)**. Taking the *FF* genotype as a reference, the odds

ratio for the risk of having any component of MS, was not significant among the different *FokI* VDR genotypes. However, pregnant women carrying the mutant homozygous *ff* genotype of the VDR *FokI* were more likely to have elevated blood pressure (OR = 1.85, 95% CI: 0.79, 4.34), low HDL (OR = 1.14, 95% CI: 0.70, 1.87), dyslipidemia (OR = 1.60, 95% CI: 0.63, 4.10), and GDM (OR = 1.08, 95% CI: 0.66, 1.76) suggesting that *ff* genotype could be a risk factor for these variables. Although we did not see statistically significant odds ratios, they were consistently in the same direction.

The prevalence of maternal obesity in Saudi women was 27%. Moreover, 12%, 29%, 63%, 55%, 34%, and 47% of participants had elevated blood pressure, GDM, hypercholesterolemia, hypertriglyceridemia, low HDL, and dyslipidemia, respectively (**Figure 6.1**).

6.4.5 MS and Its Components x Vitamin D Status

Pregnant women who had MS and carry *ff* genotype were found to have a very strong significant and inverse association between serum 25(OH)D and fasting serum glucose (r=-0.92) (**Figure 6.2**). In controls, we found a significant inverse association between serum 25(OH)D and diastolic blood pressure (DBP) regardless of genotype (r=-0.16) (**Figure 6.3**). Additionally, in all controls carrying *FF* genotype, serum 25(OH)D was significantly associated with HDL-cholesterol, LDL-cholesterol, and total cholesterol (r= 0.22, 0.19, 0.25, respectively) **(Figure 6.4)**.

Table 6.5 presents the anthropometric and biochemical characteristics of vitamin D deficient versus non-deficient subjects. Vitamin D deficiency was prevalent among 85% of our participants. Moreover, vitamin D deficient subjects had significantly higher fasting insulin, HOMA_IR and systolic blood pressure (SBP) (*P*= 0.007, 0.024, 0.032,

respectively). Whereas, HDL-C and TC were significantly higher among the non-deficient vitamin D women compared to their counterparts (*P*= 0.001, 0.049, respectively).

6.5 Discussion

To our best knowledge, this study represents the first time that the associations between the *FokI* VDR gene polymorphism and MS, as well as its components, have been evaluated in pregnant Saudi women.

We found that the *FokI* VDR polymorphism may have a strong influence on the emergence of MS. The *f* allele and the *ff* genotype seem to be risk factors for MS, while the *F* allele and the *FF* genotype appear to play a protective role. Our findings are in line with Aslani et al.,[32] who found that the frequency of the *ff* genotype was two-fold in postpartum Iranian women with MS compared to healthy participants during the postpartum follow-up (OR=2.1; 95% CI: 0.90–4.89; *P*=0.09). On the contrary, another recent study on the same population in Iran did not observe any significant associations between the *FokI* VDR polymorphism and MS in adult males nor females.[33] Results from other studies in northern China[3], the United Arab Emirates (UAE)[26], and Brazil[2] are also consistent with this finding. The variations and inconsistencies between these studies could be attributed to the environmental factors in different populations.[25] Moreover, the frequency of the VDR *FokI* genotypes and alleles differ substantially between ethnic groups and even within the same population samples,[26] as previously observed in the contradictory findings regarding the Iranian population. Such heterogeneity in allele and genotype distribution leads to varying susceptibility to diseases.[22]

There are very few studies on MS in pregnancy. In our study, the prevalence of

MS among pregnant women was 12%. Another study recently conducted in the UAE found that the prevalence of MS among adults was 14.6%.[26] Furthermore, 66% of our study population was either overweight or obese. This finding is congruent with another large cohort study performed in Saudi Arabia, in which 68% of pregnant women were found to be either overweight or obese.[9] According to a recent report by the World Health Organization (WHO), the prevalence of obesity and overweight among females aged 15 years or older in Saudi Arabia is approximately 65.9%, which is one of the highest in the Eastern Mediterranean Region, and this figure is predicted to rise to 78% by the year 2022.[34] The increasing prevalence of obesity among females in Saudi Arabia is concerning due to the myriad of adverse health outcomes it can cause in mothers and their offspring.[35] Obesity is a risk factor for many maternal and neonatal complications, including increased incidence of GDM, hypertension, cesarean-section delivery, fetal macrosomia, shoulder dystocia, and perinatal mortality.[1,36] In addition, obesity in pregnancy has a negative effect on fetal metabolic programming, thereby causing a long-term, adverse metabolic condition in the child, such as obesity, diabetes, and CVD.[36,37]

A significant body of evidence has revealed associations between obesity and vitamin D deficiency. Since vitamin D is fat-soluble, it becomes sequestered in fat cells in a form that is not bioavailable.[38] Obese individuals tend to have a higher body fat percentage, thus reducing the circulating level of serum 25(OH)D.[21,38] Another known causation linked to obesity and vitamin D deficiency is the reduction of cutaneous vitamin D synthesis as a result of decreased sunlight exposure due to clothing habits or limited outdoor activity.[39,40] In our study, 85% of pregnant women were found deficient in vitamin D (25[OH]D <50 nmol/L). This rate exceeds Al-Faris's report in 2016[24] that

50% of pregnant Saudi women living in Riyadh were vitamin D deficient, but Al-Ajlan et al.'s[41] results contained the highest rate; they found that 94% of pregnant Saudi women had vitamin D deficiency or insufficiency (25[OH]D <50 nmol/L).

Previous studies have reported a link between the *FokI* VDR polymorphism and obesity. In Egypt, Mackawy and Badawi[27] found that the *ff* genotype was associated with higher waist circumference in diabetic patients with MS, compared to the FF genotype in the same group. Similarly, Zaki et al.[21] reported higher frequencies of the mutant allele *f* in obese Egyptian women with vitamin D deficiency, compared to healthy obese women with sufficient levels of vitamin D. In their preliminary study on obese Pakistanis, Haris and Baig[42] observed a potential association between the *ff* genotype and obesity. Zhao et al.[3] also found that the *FF* genotype was associated with lower BMI, compared to the *ff* and F*f* genotypes, in Chinese adults with MS. Aslani et al.[32] also found a significant association between the *f* allele and pre-pregnancy obesity in pregnant Iranian women. In our study, however, the control-group carriers of the *FF* genotype presented higher pre-pregnancy BMI than the pregnant women carrying the F*f* and *ff* genotypes. This indicates that the *ff* genotype may be a protective factor against obesity, but the level of association did not reach statistical significance. A possible explanation for the variation between these studies could be related to ethnicity and environmental factors.

It is well known that vitamin D plays a powerful role in regulating blood pressure by suppressing renin biosynthesis for the modulation of the renin–angiotensin system (RAS).[43] Vitamin D also has anti-hypertensive properties by enhancing endothelial vasodilation as well as reducing advanced glycation products and oxidative stress.[41] We found that vitamin D deficient subjects had significantly higher systolic blood pressure

than non deficient (*P*=0.032). Similarly, a cross-sectional study found that low levels of serum 25(OH)D may be correlated with high blood pressure and increased rates of incident hypertension.[44] We also found a significant inverse correlation between serum 25(OH)D and diastolic blood pressure across all genotypes in the control group (non-MS patients) (r=-0.16). It is also notable that carriers of the mutant genotype *ff* demonstrated a higher incidence of hypertension (>130/85) compared to carriers of the homozygous and heterozygous genotypes. While these results are not statistically significant, they are consistent with Zaki et al.'s [21] findings, who reported that obese Egyptian women carrying both genotypes *Ff* and *ff* showed significantly higher blood pressure compared to those with the common homozygous genotype *FF*. Two studies found a significant association between the *FF* genotype and the risk of high blood pressure; one study linked the *FF* genotype to higher systolic blood pressure in Emirati women,[26] while the other study associated the *FF* genotype with hypertension in Indian adults.[19] Both studies attributed the cause of hypertension or elevated blood pressure to the effect of *FF* homozygotes enhancing the production of renin and angiotensin II. In contrast to these findings, Cottone et al.[45] did not detect any significant associations between blood pressure and the *FokI* VDR polymorphism, nor between blood pressure and serum 25(OH)D levels, in hypertensive Italian patients. A meta-analysis concluded that vitamin D supplementation is ineffective in lowering systolic or diastolic blood pressure and thus should not be used as an anti-hypertensive agent.[46] Such discrepancies in previous studies may be related to genetic differences in the sample populations or their exposure to different environmental factors. Until future research determines a significant association between the *FokI* VDR polymorphism and maternal hypertension, it should be stressed

that pregnant women with hypertension are at increased risk of delivering a premature baby as well as other serious complications, such as a defect in endothelial-dependent vascular function, compared to healthy pregnant women.[10] These issues should be carefully addressed when defining high risk pregnancies, and preventative measures should be undertaken.

The expression of VDR has been detected in various human tissues and cells, including adipocytes.[27] Vitamin D is known to interact with VDR in adipose tissue, thereby forming the vitamin D-VDR axis, which is important for the metabolic activation of vitamin D.[26] Any change in the axis caused by, for example, vitamin D deficiency or VDR polymorphism could affect the lipid profile through many different mechanisms.[47] Vitamin D can directly affect adipocyte differentiation and metabolism through the stimulation of parathyroid hormone secretion, thus depressing lipolysis.[39,48] Additionally, vitamin D increases intestinal calcium absorption, which reduces the formation and secretion of TG by the liver, thereby causing a decrease in serum TG levels.[49] It has also been found that vitamin D raises the concentrations of apolipoprotein A-1, which is the main protein component in HDL-C.[50] Vitamin D can also indirectly influence lipid metabolism by improving insulin secretion and sensitivity.[51] All these hypothesized mechanisms contribute to a favorable lipid profile with a decreased risk of CVD. [52]

Consistent with this notion, many studies have associated VDR gene polymorphism with dyslipidemia.[26,27,50,52] Schuch et al.[2] found that participants who did not have MS and who were carrying the mutant, homozygous genotype (*ff*) had significantly higher TG levels and lower HDL levels compared to those with the heterozygous (F*f*) or normal homozygous (*FF*) genotypes. Other authors have reached

reciprocal outcomes. Mackawy et al.[27] observed higher plasma TC, TG, and LDL-C with lower HDL-C levels in diabetic, non-MS carriers of the *ff* genotype compared to carriers of the *FF* genotype. Additionally, Hasan et al.[26] reported higher serum TC levels in Emirati carriers of the *ff* genotype compared to other genotypes of the *FokI* VDR polymorphism. These previous studies are in line with our findings, which revealed that all participants carrying the mutant, homozygous genotype were at higher risk of developing dyslipidemia compared with those carrying the normal homozygous and heterozygous genotypes. However, these results are not statistically significant. Moreover, all pregnant women with MS, regardless of their genotypes, expressed significant associations between HDL and serum 25(OH)D levels. Similarly, in the control group (non-MS), the serum 25(OH)D level was found to be significantly associated with HDL, LDL, and TC in all pregnant carrying *FF* genotypes. Surprisingly, we found that non-deficient vitamin D women had higher lipid profile compared to vitamin D deficient women. Our unexpected result was similar to the finding of Al-Ajlan et al,[41] who reported that in the subgroup of vitamin D-deficient, pregnant Saudi women, their serum vitamin D levels were significantly and positively correlated with their serum TG and TC levels. One possible explanation for this outcome, as speculated by the researchers, could be the combination of vitamin D deficiency, which was highly prevalent among the study sample, with the high metabolic demands of pregnancy.[41] It has been known that as pregnancy progresses, there is a marked increase in plasma lipid concentrations, which mimics the markers of MS.[4, 35] While increased lipid levels are considered normal physiological changes during pregnancy, maternal hyperlipidemia could cause serious health issues in the fetus by disrupting normal placentation and

damaging the vessel wall via increased oxidative stress.[11] Previous studies have shown a strong relationship between hyperlipidemia and increased incidence of preterm birth, which is considered a leading cause of prenatal morbidity and mortality.[53,54]

As previously mentioned, the presence of VDR in human tissue, including pancreatic β-cells, inspired many researchers to study the association between VDR gene polymorphism and glucose homeostasis.[26,32] In Brazil, Schuch et al.[2] observed significantly higher β-cell secretion (HOMA-β) in individuals carrying the *f* allele than those carrying the F allele. Both Schuch et al.[2] and Zaki et al.[21] reported a significant association between the *ff* genotype and a higher HOMA-IR, compared to the *FF* and *Ff* genotypes of the *FokI* VDR polymorphism, in Brazilian and Egyptian populations, respectively. Similarly, Mackawy et al.[27] found that diabetic Egyptian patients with MS who carried the *ff* genotype presented higher insulin levels and HOMA-IR than those with the *FF* and *Ff* genotypes. These outcomes support our finding of a statistically significant and inverse correlation between serum 25(OH)D levels and fasting serum glucose in pregnant women carrying *ff* genotype in MS group.

There are very few studies available that examined the effect of the *FokI* VDR polymorphism on GDM. A link between the *FokI* VDR polymorphism and GDM risk was conducted among Iranian[32] and Saudi populations,[55] with contradictory results. In Iran, Aslani et al.[32] found that the frequency of the *ff* genotype in GDM patients was higher than in normal pregnant women (10.6% vs. 6.2%). A higher frequency of the *F* allele was also observed in healthy subjects (P=0.06), suggesting that the *F* allele may represent a protective factor against GDM.[33] In Saudi Arabia, El-Beshbishy et al.[55] also found a higher frequency of the F allele (56.4% vs. 35.7%) and a lower frequency of the *f*

allele (43.6% vs. 64.3%) in their control group compared to their GDM group, but the results were not statistically significant (*P*=0.100). The authors concluded that there was no significant association between the *FokI* VDR polymorphism and GDM in the Saudi population.[55] The present results agree with El-Beshbishy et al.,[55] as we did not observe any statistically significant associations between GDM and the *FokI* VDR polymorphism. However, a logistic regression analysis revealed that carriers of the *ff* genotype are at higher risk of developing GDM, compared to carriers of other genotypes of the *FokI* VDR polymorphism (odds ratio=1.27; 95% CI: 0.76–2.14; *P*=0.360, after adjustments for age and BMI). In support of the present findings, Al-Daghri et al.[56] found a statistically significant association between the *F* allele and a reduced risk of developing T2DM among the Saudi population (*P*=0.02). It is well known that *GDM mimics* T2DM in its pathology, as both conditions cause defects in insulin secretory response.[57] We speculate that a larger cohort study with sufficient statistical power is necessary to affirm the potential of VDR genetic variation in predicting GDM.

The pathophysiological mechanisms of the previous associations are still unclear. It has been hypothesized that vitamin D plays a role in glucose homeostasis through several underlying pathways; for example, the active hormonal metabolite of vitamin D (1,25-dihydroxyvitamin D) binds to the VDRs of pancreatic β-cells, which stimulates insulin secretion and regulates the balance between the extracellular and intracellular calcium pools.[57] Thus, calcium is an important co-factor for insulin-mediated intracellular functions in insulin-responsive tissues, as any changes in intracellular calcium may cause peripheral insulin resistance.[58] In addition, the VDR affects glucose homeostasis via the insulin-like growth factor system;[23] any genetic changes in the VDR might contribute to

the pathogenesis of GDM via alterations in calcium metabolism and modulation of insulin secretion.[27] GDM can have serious consequences with lasting metabolic changes for both mother and offspring.[1] Long-term cohort studies have reported that women with GDM have an 35–65% increased risk of developing T2DM later in life, and their offspring are more likely to develop obesity and T2DM in childhood and adulthood.[1,57]

In our study, vitamin D deficiency was evident in 85% of the sample population. Our results are in line with Al Foda et al.[59] and Al-Sheikh et al.[60] who reported a high prevalence of vitamin D deficiency amounting to 86%. We did not detect any significant difference between MS and controls in regards to vitamin D deficiency, as both groups had high prevalence of vitamin D deficiency.

We acknowledge that there are some limitations in our study. Due to the limited frequency of mutant *ff* genotype, we were not able to observe an association between *FokI* and components of MS. We strongly believe for future prospective studies, a sufficient statistical power is vitally needed, especially when studying the effect of mutant *ff FokI* genotype on MS and its components. In addition, we analyzed the VDR gene at only one SNP—albeit one that translates structurally diverse VDR proteins, which vary in their potential to elicit vitamin D-mediated gene expression. Unfortunately, we did not control for confounding factors such as dietary habits, physical activity or other lifestyle factors, which may impact the risk of MS. Despite these limitations, and to our best knowledge, this study is the first to examine the relationships between the *FokI* VDR polymorphism and MS, as well as its components, among pregnant Saudi women; in fact, no previous study has investigated such associations in pregnancy among any population. Accuracy can also be assured in our vitamin D measurements, as we

investigated the role of vitamin D status and correlated it with different variables related to the components of MS in all *FokI* genotypes. Unlike previous epidemiological studies, detailed and valid data for each MS component, such as blood pressure, and the fasting serum samples, including the lipid, glucose, and insulin concentrations, were precisely measured and not self-reported.

6.6 Conclusion

The past few years have seen dramatic changes to the lifestyle of Saudi Arabians, which have led to notable increases in the prevalence of MS, especially among pregnant women, associated with an increased incidence of CVD, T2DM, GDM, and dyslipidemia in the Saudi population. Despite this, there is a marked lack of research on the genetic risk factors, such as VDR gene polymorphism, in MS occurrence among this population, and no study has examined such associations during pregnancy.

To address this issue, we conducted a study to observe the effect of the *FokI* VDR polymorphism on the pathology of MS and its components among pregnant Saudi women. We found a statistically significant association between the *FokI* VDR polymorphism, specifically in carriers of the *ff* genotype, and an increased risk of MS among the sample. This association could be a prognostic tool for predicting the risk of developing MS in pregnancy.

Knowledge of the associations between the *FokI* VDR polymorphism and the risk of MS is crucial for many reasons. One is that the early diagnosis of MS during pregnancy allows for the identification of patients who are at risk of developing CVD and other adverse metabolic changes.[61] Another reason is that early intervention, such as

vitamin D supplementation, can be used to treat this condition and mitigate its adverse effects on mothers and their offspring.

The results of our study suggest that the *FokI* VDR polymorphism may affect the components of MS, namely obesity, dyslipidemia, and GDM, but the correlation is not statistically significant. Our findings warrant further investigations using larger cohorts as well as future associative studies on other VDR gene polymorphisms.

Table 6.1 Demographic and biochemical characteristics of MS vs. normal subjects

Parameters	All	Metabolic Syndrome	Control	*P*-value*	*P*-value**
N	368	44 (12.0)	324 (88.0)		
Age (years)	**29.1±5.6**	**31.3±6.9**	**28.7±5.3**	**0.02**	
Pre-pregnancy BMI (kg/m^2)	**26.9±5.9**	**33.3±4.1**	**25.9±5.5**	**<0.001**	
Current BMI (kg/m^2)	**28.2±6.2**	**34.7±4.5**	**27.5±5.9**	**<0.001**	0.956
Parity	2 (1-4)	2(1-5)	2(1-4)	0.210	0.539
SBP (mmHg)	113.3±12.9	119.1±15.7	112.3±12.1	0.020	0.272
DBP (mmHg)	**66.9±9.2**	**71.7±11.4**	**66.1±8.5**	**0.010**	**0.030**
HbA_{1C} (%)	**4.8±0.5**	**5.1±0.7**	**4.8±0.8**	**<0.001**	**0.005**
FBG (mmol/L)	**4.6 ± 1.0**	**5.6±1.5**	**4.5±0.8**	**<0.001**	**<0.001**
HOMA_IR	**1.5 (0.9-2.5)**	**3.1 (1.7-5.2)**	**1.3 (0.9-2.3)**	**<0.001**	**<0.001**
Insulin (uU/ml)	**7.5 (4.5-13.1)**	**14.2(7.9-22.8)**	**6.9(4.1-11.9)**	**<0.001**	**0.003**
HDL-C (mmol/L)	**1.5±0.4**	**1.3±0.5**	**1.6±0.4**	**<0.001**	**<0.001**
LDL-C (mmol/L)	3.9±1.3	3.9±1.4	3.9±1.3	0.698	0.269
TC (mmol/L)	6.2±1.2	6.4±1.3	6.2±1.5	0.271	0.084
TG (mmol/L)	**1.8(1.4-2.3)**	**2.6(2.1-3.4)**	**1.7(1.3-2.2)**	**<0.001**	**<0.001**
25(OH)D (nmol/L)	33.4(21.3-53.7)	29.9(17.9-44.6)	33.7(21.7-54.4)	0.232	0.186
Vitamin D deficiency (<50nmol/L)	314 (85.3)	38 (86.4)	276 (85.2)	0.291	0.199

Note: Pre-preg BMI: Pre-pregnancy body mass index; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; FBG: fasting blood glucose; HbA_{1C}: haemoglobin A1c or glycated haemoglobin; HDL: High-density lipoprotein Cholesterol; LDL: low-density lipoprotein Cholesterol; TC: Total Cholesterol, TG: Triglycerides. Data Presented as Mean ± SD and Median (25th -75th) percentiles for Gaussian and Non Gaussian variables. **P*-value adjusted for age & pre-pregnancy BMI; significant at 0.05 and 0.01 levels.

Table 6.2 The genotype distribution of VDR *FokI* polymorphisms in MS and control groups

***FokI* Genotype**	**All**	**MS**	**Control**	**β**	**OR (95 % CI)**	***P*-Value**	**Adjusted OR**	** *P*-Value**
FF	225 (61.1)	22 (50.0)	203 (62.7)		1		1	
Ff	118 (32.1)	16 (36.4)	102 (31.5)	0.37	1.45 (0.73-2.88)	0.291	1.42 (0.71-2.9)	0.307
ff	**25 (6.8)**	**6 (13.6)**	**19 (5.9)**	**1.07**	**2.91 (1.05-8.1)**	**0.039**	**4.17 (1.42-12.2)**	**0.009**
Ff+ff	143 (38.9)	22 (50.0)	121 (37.3)	0.52	1.68 (0.89-3.16)	0.109	1.76 (0.92-3.36)	0.090
F	568 (77.2)	60 (68.2)	508 (78.4)		1		1	
f	**168 (22.8)**	**28 (31.8)**	**140 (21.6)**	**0.53**	**1.69 (1.04-2.75)**	**0.034**	**1.84 (1.05-1.14)**	**0.017**

Note: ORs: Odds ratio (95% CI); **P*-value significant at < 0.05, ***P*-value adjusted for age and significant at <0.05,0.01

Table 6.3 Clinical characteristics of the *FokI* VDR genes polymorphisms in MS and control groups

Parameters	***FF***	***Ff***	***ff***	***P*-value**
Metabolic Syndrome				
N	22	16	6	
Age (years)	29.1±5.4	28.5±5.3	26.8±3.6	0.190
Pre-preg BMI (kg/m^2)	34.2±4.8	33.3±2.6	30.3±4.3	0.127
Current BMI (kg/m^2)	35.3±4.8	34.8±3.1	32.6±6.3	0.430
Parity	2.0(1.0-5.0)	3.0(1.0-7.0)	4.0(2.0-6.0)	0.635
SBP (mmHg)	111.9±12.3	112.9±11.4	112.3±14.2	0.870
DBP (mmHg)	65.6±8.3	67.2±9.1	65.3±7.9	0.471
HbA_{1C} (%)	4.7±0.4	4.8±0.6	4.8±0.4	0.645
FBG (mmol/L)	4.5±0.9	4.4±0.7	4.5±0.3	0.594
HOMA_IR	3.7 (1.9-5.3)	3.7(1.7-5.9)	2.1(1.1-2.7)	0.132
Insulin (uU/ml)	7.4 (4.1-12.3)	6.4 (4.2-10.9)	6.4 (3.6-17.9)	0.907
HDL-C (mmol/L)	1.6±0.4	1.5±0.4	1.6±0.4	0.949
LDL-C (mmol/L)	3.8±1.3	3.9±1.2	3.7±1.0	0.749
TC (mmol/L)	6.2±0.6	6.2±1.4	6.2±1.2	0.996
TG (mmol/L)	2.7 (2.1-3.2)	2.5 (2.2-3.4)	3.3 (2.2-3.4)	0.761
25(OH)D (nmol/l)	29.9 (18.0-46.9)	37.3(20.4-60.6)	22.2(15.7-28.3)	0.234
Control				
N	203	102	19	
Age (years)	32.4±7.1	31.8±6.6	26.3±5.5	0.144
Pre-pregnancy BMI (kg/m^2)	26.6±5.9	24.7±4.3	25.9±5.7	**0.021**
Current BMI (kg/m^2)	27.9±6.3	25.9±4.7	27.0±5.9	**0.027**
Parity	2.0(1.0-4.0)	2.0(1.0-3.0)	1.0(1.0-2.0)	0.439
SBP (mmHg)	120.1±15.3	119.3±15.5	112.0±22.5	0.720
DBP (mmHg)	70.8±11.6	73.4±10.3	69.7±18.5	0.796

HbA_{1C} (%)	5.1±0.6	5.0±0.8	5.2±0.9	0.840
FBG (mmol/L)	5.4±1.5	6.2±1.6	5.0±0.9	0.156
HOMA_IR	1.4 (0.8-2.3)	1.2 (0.8-2.1)	1.3 (0.8-3.6)	0.616
Insulin (uU/ml)	16.2 (9.3-22.7)	15.5 (6.7-24.7)	7.9 (6.0-11.7)	0.081
HDL-C (mmol/L)	1.3±0.5	1.4±0.5	1.2±0.2	0.802
LDL-C (mmol/L)	4.1±1.6	3.7±1.2	4.1±0.9	0.744
TC (mmol/L)	6.4±1.3	6.4±1.4	6.6±0.9	0.922
TG (mmol/L)	1.7 (1.3-2.2)	1.7 (1.2-2.2)	1.6 (1.4-2.3)	0.729
25(OH)D (nmol/l)	34.3(23.2-54.5)	31.4(18.8-52.1)	40.4(23.2-85.1)	0.090

Note: Data represented Mean ± SD and Median (25^{th} and 75^{th}) percentile for Gaussian and non-Gaussian variables. *P*-value significance at 0.05.

Table 6.4 Risk predictions for different genotypes and alleles of *FokI* VDR gene polymorphisms in relation to metabolic syndrome and its components

Parameters	Yes	β	OR (95 % CI)	P-Value
Obesity (≥ 30 kg/m^2)	101			
FF	66 (65.3)		1	
Ff	28 (27.4)	0.030	1.03 (0.40-2.63)	0.950
ff	7 (7.4)	-0.363	0.70 (0.41-1.18)	0.179
Ff+ff	35 (34.8)	-0.291	0.75 (0.46-1.22)	0.245
F	160 (28.3)		1	
f	42 (25.1)	-0.169	0.84 (0.56-1.27)	0.844
Elevated Blood Pressure (> 130/85)	44			
FF	19 (46.2)		1	
Ff	19 (46.2)	0.464	1.59 (0.32-7.89)	0.571
ff	6 (7.7)	0.616	1.85 (0.79-4.34)	0.157
Ff+ff	25 (53.9)	0.592	1.81 (0.79-4.11)	0.157
F	57 (10.3)		1	
f	31 (14.7)	0.409	1.51 (0.80-2.83)	0.205
Low HDL-C (< 1.3 mmo/l)	126			
FF	76 (60.0)		1	
Ff	42 (33.0)	0.072	1.07 (0.44-2.66)	0.877
ff	8 (7.0)	0.133	1.14 (0.70-1.87)	0.595
Ff+ff	50 (40.0)	0.122	1.13 (0.71-1.79)	0.604
F	194 (33.8)		1	
f	58 (35.8)	0.087	1.09 (0.75-1.59)	0.652

Hypertriglyceridemia (> 1.7 mmol/l)	202			
FF	127 (62.8)		1	
Ff	60 (29.6)	0.131	1.14 (0.49-2.65)	0.761
ff	15 (7.5)	-0.240	0.79 (0.50-1.23)	0.297
Ff+ff	75 (37.1)	-0.175	0.84 (0.55-1.28)	0.419
F	314 (55.6)		1	
f	90 (53.6)	-0.079	0.92 (0.65-1.31)	0.656
Hypercholesterolemia (> 5.7 mmol/l)	233			
FF	137 (58.5)		1	
Ff	78 (33.6)	0.501	1.65 (0.66-4.12)	0.283
ff	18 (7.9)	0.237	1.27 (0.79-2.03)	0.324
Ff+ff	96 (41.5)	0.282	1.33 (0.85-2.07)	0.214
F	352 (62.1)		1	
f	114 (68.1)	0.265	1.31 (0.90-1.89)	0.158
Dyslipidemia	171			
FF	88 (51.5)		1	
Ff	69 (40.5)	-0.004	1.00 (0.57-1.75)	0.324
ff	14 (8.0)	0.470	1.60 (0.63-4.10)	0.989
Ff+ff	83 (48.5)	0.088	1.09 (0.65-1.84)	0.740
F	245 (71.6)		1	
f	97 (29.4)	0.101	1.11 (0.72-1.71)	0.648
GDM	108			
FF	65 (60.2)		1	
Ff	36 (33.3)	-0.04	0.96 (0.38-2.40)	0.926
ff	7 (6.5)	0.06	1.08 (0.66-1.76)	0.755
Ff+ff	43 (39.8)	0.06	1.06 (0.66-1.68)	0.808
F	166 (76.9)		1	
f	50 (23.1)	0.03	1.03 (0.70-1.50)	0.704

Note: Data Represented unstandardized β, odd ratio and (95 % CI*). P*-value significant at < 0.05.

Table 6.5: Clinical characteristics of the vitamin D deficient vs. non-deficient pregnant Saudi women

Parameters	Vitamin D deficient 25(OH)D<50 nmol/l	Non-deficient 25(OH)D >50 nmol/l	P-value
N	**260 (85.3)**	**108 (14.7)**	
Age (years)	28.9 ± 5.7	29.4 ± 5.2	0.470
Pre-preg BMI (kg/m^2)	26.8 ± 6.1	26.9 ± 5.5	0.852
Current BMI (kg/m^2)	28.2 ± 6.5	28.1 ± 5.6	0.835
Parity	2.0 (1.0-4.0)	1.0 (1.0-3.0)	0.202
SBP (mmHg)	114.6 ± 12.9	110.8 ± 12.4	**0.032**
DBP (mmHg)	67.7 ± 9.1	65.5 ± 9.2	0.070
HbA_{1C} (%)	6.3 (5.3-7.4)	6.8 (5.6-9.1)	0.756
FBG (mmol/L)	4.5 (4.1-4.9)	4.2 (3.9-5.1)	0.081
HOMA_IR	7.6 (4.6-13.1)	6.3 (4.5-12.9)	**0.024**
Insulin (uU/ml)	1.5 (0.9-2.5)	1.3 (0.9-2.7)	**0.007**
HDL-C (mmol/L)	1.5 ± 0.4	1.6 ± 0.4	**0.001**
LDL-C (mmol/L)	3.8 ± 1.3	3.9 ± 1.3	0.671
TC (mmol/L)	6.1 ± 1.5	6.4 ± 1.4	**0.049**
TG (mmol/L)	1.80(1.4-2.3)	1.90 (1.5-2.4)	0.684
25(OH)D (nmol/l)	29.9 (20.1-46.7)	66.7 (43.4-85.3)	**<0.001**

Note: Data represented Mean ± SD and Median (25^{th} and 75^{th}) percentile for Gaussian and non-Gaussian variables. *P*-Value Denotes significance at P <0.05 and 0.01.

Figure 6.1 Presence of metabolic disorders among pregnant Saudi women in their second trimester

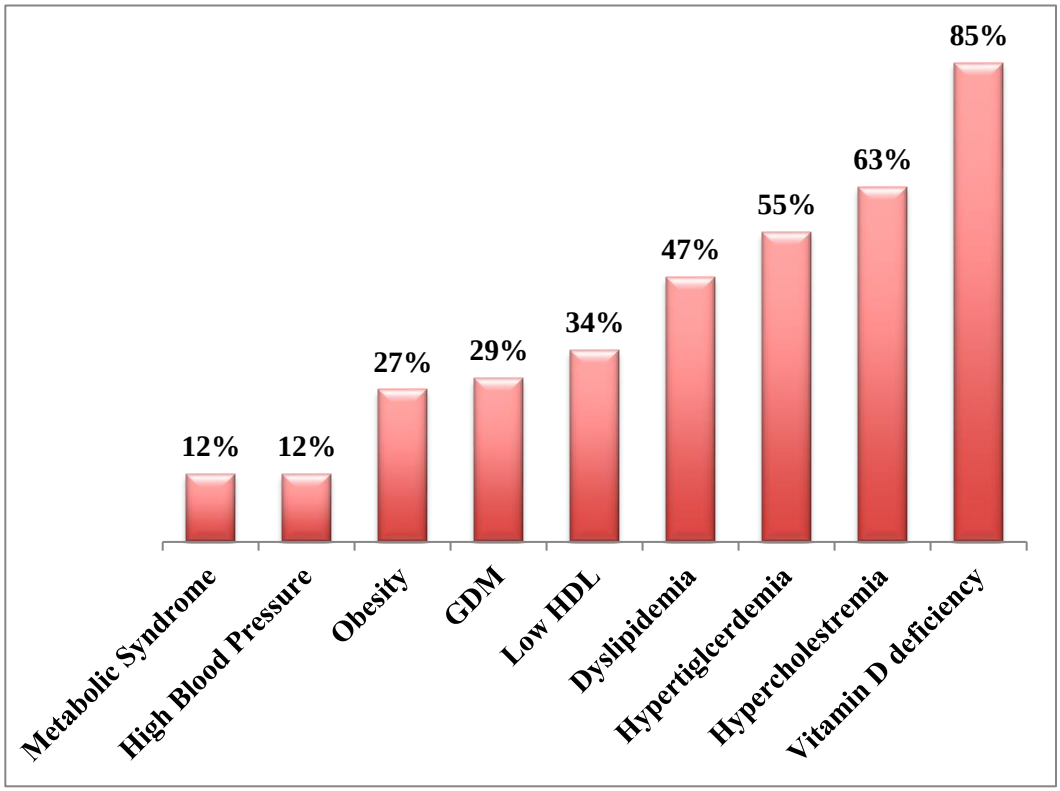


Figure 6.2 Correlation between Log vitamin D (nmol/L) in *ff FokI* genotypes versus FBG in MS subjects

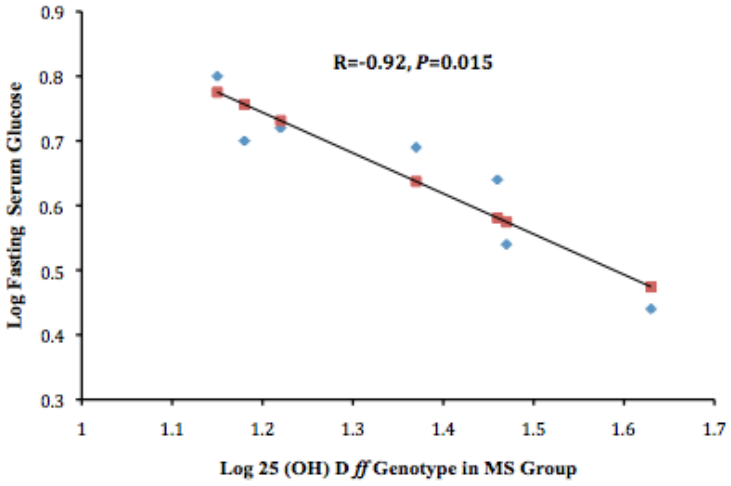


Figure 6.3 Correlation between Log vitamin D (nmol/L) and diastolic blood pressure (DBP) in controls, regardless of their *FokI* genotype

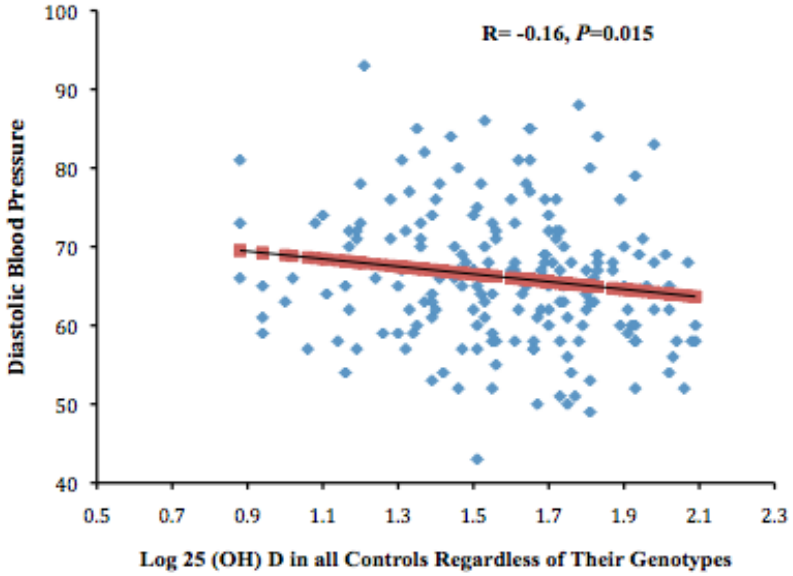
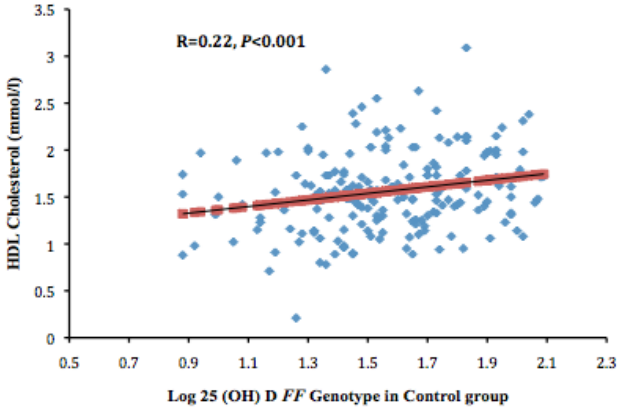
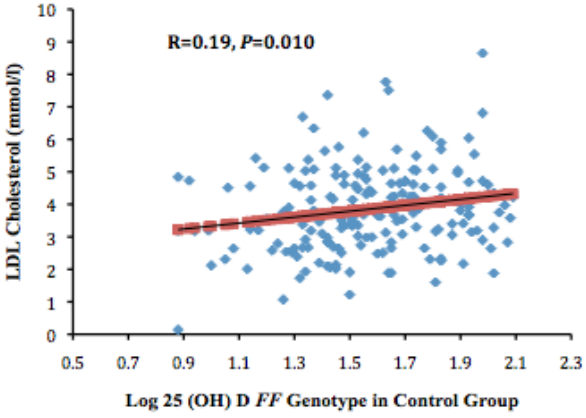


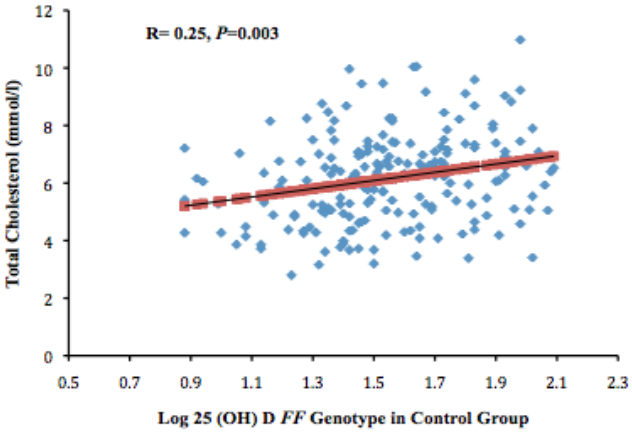
Figure 6.4 Correlations between Log vitamin D (nmol/L) in *FF FokI* genotypes versus (A) HDL-C, (B) LDL-C, (C) TC in controls



A



B



C

6.7 References

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CHAPTER 7

CONCLUSION AND FUTURE DIRECTIONS

7.1 Conclusion

This is the first study to examine the metabolic impact of the *ff* VDR genotype versus the *FF* wild type VDR in pregnant Saudi women, who are known to be vitamin D deficient. We thoroughly investigated the genetic influence of *FokI* VDR polymorphism on vitamin D deficiency, gestational diabetes, glucose intolerance, and metabolic syndrome while controlling for classic confounders that may mask the findings of our results. Our findings are important and encouraging because, if replicated and proved correct, they will advance the medical field by determining the possible culprit or predictor of adverse outcomes for both mother and infant. This new knowledge is significant because it will lead to significant changes in current obstetric and pediatric practice, facilitating early identification of any at-risk pregnancy and supplementing pregnant women with vitamin D. This in turn will reduce the risk of morbidity and mortality as well as reduce the economic burden of these chronic diseases. The following section highlights our major research results.

- We have successfully identified unique predictors according to the following health problems Saudi women face during pregnancy:
    - **Vitamin D Deficiency**: *FokI FF* genotype and the *F* allele might be important predictors of an individual's susceptibility to vitamin D deficiency in pregnant Saudi women, thus leaving them unprotected and at risk of developing adverse skeletal and non-skeletal health problems.

Other identified risk factors for vitamin D deficiency in our study sample included full-body clothing coverage, lower levels of physical activity, residence in areas other than North Riyadh, working indoors, and the amount of sunlight exposure.

- **Gestational Diabetes (GDM):** vitamin D deficiency appeared to be a major risk for GDM in Saudi women. Although we did not detect any association between *FokI* VDR gene polymorphism and GDM, we found that the glucose-lowering effects of a higher vitamin D level may be dependent upon the *FokI* genotype within the Saudi population. Other identified risk factors for GDM in our study sample included previous history of GDM, fasting insulin, HOMA_IR, and obesity.
- **Metabolic Syndrome (MS):** Our results indicate that the presence of the *ff FokI* VDR genotype is a genetic marker for MS risk in pregnant Saudi women. However, we did not find any significant association between *FokI* VDR genotype and components of MS in our subjects. This meaningful association could be a potential prognostic tool for predicting development of MS in pregnancy.

❖ We have observed an alarmingly high prevalence of the following metabolic disorders in Saudi women:

- Vitamin D deficiency was prevalent among 79% of pregnant Saudi women in their first trimester. Full-body clothing coverage, lower levels of physical activity, residence in areas other than North Riyadh, working

indoors, and the amount of exposure to sunlight were found to be major risk factors for vitamin D deficiency in pregnant Saudi women.

- We noticed that in the second trimester, the prevalence of vitamin D deficiency increased to 85%.
- Using IADPSG criteria, we found that about 29% of our participants were diagnosed with GDM. This rate is extremely high when compared to the overall prevalence of GDM (17.8 %) found in the *Hyperglycemia and Adverse Pregnancy Outcome **(**HAPO**)*** cohort using the same diagnostic criteria as ours.
- Metabolic Syndrome was evident in 12% of our study population. To the best of our knowledge, no study has examined the prevalence of MS in pregnancy.
    - Prevalence of the components of MS: 66% of our study population were either overweight or obese. Moreover, 63%, 55%, 34% of participants had hypercholesterolemia, hypertriglyceridemia, and low HDL, respectively.

7.2 Future Directions

Based on our study results, we believe that a more thorough understanding of the association between *FokI* VDR gene polymorphism and metabolic health disorders during pregnancy, especially in a population with a high rate of vitamin D deficiency, is vital to improving current diagnoses and intervention strategies for both mother and child. Therefore, we recommend the following be addressed in future research:

- Our study indicates the need for sufficient statistical power, especially when studying the effect of mutant *ff FokI* genotype. Unlike the wild *FF FokI* genotype, mutant *ff FokI* genotype has a limited frequency, thus the role of genotype *ff* is still unclear. The results of our study suggest that *FokI* VDR gene polymorphism may influence MS components, namely obesity, dyslipidemia, and GDM, although the correlation was not statistically significant. Our findings warrant further investigation in larger cohorts to confirm the potential association. We believe that we could have observed a significant association between *FokI* VDR genotype and components of MS if the frequency of mutant *ff* were high.
- Further research is warranted for investigating the association between *FokI* VDR genotype polymorphism and metabolic outcomes in pregnancy, with substantial need to adjust for important classical and non-classical confounders that are known to be risk factors for GDM, vitamin D deficiency, and MS. Controlling for such covariates will reveal the independent effects of genotype on adverse perinatal outcomes, if in fact any association truly exists.
- We call for interventional trials such as supplementing pregnant women, especially deficient subjects, with high and safe doses of vitamin D while studying the responsiveness of different VDR genotypes. These kinds of trials are important as they help to better identify which genotype carrier will benefit the most from supplementation. Because we assume that carriers of the mutant *ff* genotype are considered high-risk patients, or "low responders," they need higher doses of vitamin D supplements than non-carriers to achieve sufficient levels and benefit the most from treatment.

- Further studies on healthy subjects are required to identify the genetic predictors of 25(OH)D concentrations, as most of the research that investigated such an association was conducted with patients who had different diseases. There is a possibility that a specific disease could have masked the genetic influence of VDR on vitamin D status.
- Through our literature review, we noticed that there are numerous genes involved in the vitamin D metabolic pathway, aside from VDR gene, that significantly impact vitamin D status and are worth studying. Vitamin D binding protein as well as *25-Hydroxyvitamin D-24-hydroxylase* (*CYP24A1*) are among those genes that showed significant association with vitamin D status.
- We would like to shed light on a neglected issue yet to be assessed. No studies to date have investigated the genetic risk factors, such as VDR gene polymorphism, on the occurrence of MS during pregnancy. Examining such a relationship is vital for preventing short- and long-term adverse effects on both mother and baby.
- Saudi Arabia has a young population, with more than half of the population younger than 25 years. Therefore, research is needed among this age group to develop strategies for primary care intervention. If these current high rates of vitamin D deficiency, GDM, obesity, MS, and dyslipidemia that we observed in our population are not reversed early in life, the future disease burden will soar in Saudi Arabia.
- No genome-wide association studies (GWAS) have been conducted in Saudi Arabia. These kinds of studies are important for the detection of novel genetic variants that may play a role in the occurrence of adverse health outcomes in the

Saudi population. Understanding the pathophysiological mechanisms of genes related to the vitamin D metabolic pathway might bring insight into the genetic components of different diseases in the Saudi population, especially in women of child-bearing age.

APPENDIX A

ETHICAL APPROVALS

Kingdom of Saudi Arabia
Ministry of Higher Education
King Saud University
Code 034
College of Medicine
& King Khalid University Hospital

بسم الله الرحمن الرحيم

المملكة العربية السعودية
وزارة التعليم العالي
جامعة الملك سعود
رمزها ٠٣٤
كلية الطب
ومستشفى الملك خالد الجامعي

Date: 11.02.2014 التاريخ: 11.04.1435 No.: 14/4067/IRB الرقم:

To : **Dr. Abdulrahman Al Ajlan**
Riyadh College of Health Sciences

Re : **Research Project No. E-13-1013**
"Prevalence of Vitamin D deficiency in pregnant women and its association with gestational diabetes mellitus"

Dear Dr. Al Ajlan,

Thank you for your response to the comments raised by the Board regarding the above-mentioned research project, which was reviewed and discussed in the IRB Meeting 4 (Academic Year 1434-1435) held on 26 December 2013 (23 Safar 1435). The IRB has reviewed your response and found that you have answered satisfactory and adequately fulfilled the requirements. Therefore, the project is now **approved**. Work on this project may begin.

We wish you success in your research and request you to keep the IRB informed about the progress of the study on a regular basis by submitting a *Study Progress Report* every 6 months and a *Final Report* when the study has been completed. If you make any changes to the protocol, you must submit a revised protocol to the IRB for approval before implementing the changes.

Thank you!

Sincerely yours,

Dr. Khalid M. Al-Faleh
Chairman, Institutional Review Board
King Saud University - College of Medicine
Email: kfaleh@ksu.edu.sa

/rubie

P. O. Box 7805, Riyadh 11472 Tel 4670011 Fax.: 4672439 ص .ب ٧٨٠٥ الرياض ١١٤٧٢ هاتف: ٤٦٧٠٠١١ فاكس: ٤٦٧٢٤٣٩

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Kingdom of Saudi Arabia
Ministry of Health
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مدينة الملك فهد الطبية
King Fahad Medical City

IRB Registration Number with KACST, KSA: H-01-R-012
IRB Registration Number with OHRP/NIH, USA: IRB00008644
Approval Number Federal Wide Assurance NIH, USA: FWA00018774

April 21, 2012
IRB Log Number: 12-081
Category of Approval: **EXPEDITED**

Dear Dr. Aisha Mansoor Ali,

I am pleased to inform you that your study titled: '**Prevalence of Vitamin D Deficiency in Pregnant Women and its association with Gestational Diabetes Mellitus (GDM)**' was reviewed and was approved.

Please be informed that in conducting this study, you as the Principal Investigator are required to abide by the rules and regulations of the Government of Saudi Arabia and KFMC/IRB. Further, you are required to submit a Progress Report before 21 March 2013; it can be reviewed by the IRB without lapse of approval. The approval of this proposal will automatically be suspended on 21 April 2012 pending the acceptance of the Progress Report. You also need to notify the IRB as soon as possible in the case of:

1. Any amendments to the project;
2. Termination of the study;
3. Any serious unexpected adverse events (within two working days).
4. Any event or new information that may affect the benefit/risk ratio of the proposal.

Please observe the following:

1. Personal identifying data should only be collected when necessary for research;
2. The data collected should only be used for this proposal;
3. Data should be stored securely so that a few authorized users are permitted access to the database;
4. Secondary disclosure of personal identifiable data is not allowed.
5. Copy of the Consent Form should be kept in the Research Subject's Medical Record and the consent process should be documented in the medical record.

المرفقات : .....

الرقم : .....
التاريخ : .....

Kingdom of Saudi Arabia
Ministry of Health
King Fahad Medical City

وزارة الصحة
مدينة الملك فهد الطبية
King Fahad Medical City

المملكة العربية السعودية
وزارة الصحة
مدينة الملك فهد الطبية

We wish you every success in your research endeavor.

If you have any further questions feel free to contact me.

Thank you.

Sincerely Yours,

F M C
Approved
21 APR 2012
I R B

Prof. Omar Hassan Kasule
Chairman - Institutional Review Board (IRB)
King Fahd Medical City
Riyadh, KSA
Tel: + 966 1 288 9999 Ext. 7540
E-mail: okasule@kfmc.med.sa

APPENDIX B

CONSENT FORM

Consent form	إقرار بالموافقة على المشاركة في الدراسة

هذا مشروع مدعوم من قبل الخطة الوطنية للعلوم والتقنية برقم

12-MED2504-02

Title of the study:

Prevalence of Vitamin D Deficiency in Pregnant Women and its Association with Gestational Diabetes Mellitus (GDM)

Aim of the study:

- To determine the prevalence of vitamin D deficiency in pregnant Saudi women
- To look for an association between vitamin D status and the incidence of gestational diabetes mellitus (GDM)
- To determine dietary vitamin D intake and calcium intake and associate it to the indices of obesity and GDM in Saudi pregnant women.
- To evaluate the influence of vitamin D status on foetal growth and development, as measured by foetus weight and fetal growth rate.

I agree on the following:

- The doctor in charge has explained the study
- If I have questions or fears I can call the doctor or his assistant any time during the study.
- They can go through my medical records in relation to the study providing full confidentiality of my information.
- I know that participating in this study is by volunteer.
- Patient has the right to be withdrawn from the study at any time without mentioning reasons.
- Patient has the right in medical care and treatment
- I agree to participate in this study and I understood this agreement form and I will sign accordingly.

عنوان الدراسة:

انتشار نقص فيتامين (د) لدى النساء الحوامل وارتباطه مع مرض سكر الحمل (GDM)

الهدف من الدراسة:

- ١. تحديد مدى انتشار نقص فيتامين (د) في النساء السعوديات الحوامل
- ٢. البحث عن وجود ارتباط بين حالة فيتامين (د) وحدوث مرض السكري الحمل (GDM).
- تحديد كمية فيتامين د والكالسيوم المتناوله وربطه بمؤشرات السمنة وداء السكري أثناء الحمل في النساء الحوامل السعودية.
- تقييم تأثير حالة فيتامين Dعلى نمو الجنين وتطوره بقياس وزن الجنين ومعدل نموه.

أقر أنا الموقع أدناه:

- أن الطبيب المسؤول شرح لي عن كيفية الدراسة
- إذا كانت لدي أي مخاوف أو أسئلة أخرى فإنني أستطيع الإتصال بالطبيب المعالج أو من ينوب عنه طوال فترة الدراسة .
- أوافق على أن من الممكن مراجعة سجلاتي الطبية ضمن إطار هذه الدراسة شريطة التأكد من أن هويتي الشخصية ستبقى سرية تماما في جميع الأوقات .
- إنني أدرك أن الإشتراك طوعي في الدراسة.
- يحق للمشارك الانسحاب من الدراسة في اي وقت بدون ذكر الأسباب.
- يحق للمشارك الاهتمام من الرعاية الطبية والعلاج عند المخاطر.
- وأفهم تماما نموذج الموافقة وما فيه من معلومات ، وبناءا على ذلك أوقعه طوعا وبملئ إرادتي.

Participant name: ……..

Signature: ……

Date:……

In charge doctor.: ……

Signature: ……

Date: ……

إسم المشترك : .....

التوقيع : .....

التاريخ: .....

إسم الطبيب المسؤول : .....

التوقيع: .....

التاريخ : .....

APPENDIX C

QUESTIONNAIRES

First and Second visit questionnaires

<table>
<tr><td colspan="3"></td></tr>
<tr><td>Research code:</td><td>Date of filling of the form:</td><td>Date of filling of the form:</td></tr>
<tr><td>Name:
Tel or Mobile No:</td><td>Place of birth:
Week of gestation……</td><td>Date of Birth (age):</td></tr>
<tr><td colspan="3"><u>Mother anthropometrics</u>
❖ Ht.....cm Current Wt.....kg
Prepregnancy Wt..... kg
Current BMI……. kg/ m2
Before pregnancy BMI……. kg/ m2

❖ Blood Pressure:.....</td></tr>
<tr><td colspan="3">Sociodemographic Measurements</td></tr>
<tr><td>Education Level</td><td colspan="2">☐Illiterate ☐Primary ☐Intermediate ☐Secondary ☐University ☐Post graduate</td></tr>
<tr><td>Family Income Per month</td><td colspan="2">☐No Income ☐less than 5000 S.R ☐ 5000-10000 S.R
☐10000-20000 S.R ☐ More than 20000 S. R</td></tr>
<tr><td>Occupation</td><td colspan="2">☐ House wife ☐Retired ☐Student ☐ Teacher
☐Employee ☐Physician ☐Businesswomen ☐Other (……)</td></tr>
</table>

Marital status	☐ Married ☐Single ☐Divorced ☐Widow
Does your husband a relative?	☐Yes ☐No ☐Relative degree ☐First degree ☐Second degree
Living area	☐ North Riyadh ☐West Riyadh ☐East Riyadh ☐South Riyadh ☐Center Riyadh
Skin color	☐ North Riyadh ☐West Riyadh ☐East Riyadh ☐South Riyadh ☐Center Riyadh

Clinical Measurements

Medical history: Family

☐HTN ☐Hyperlipidemia ☐ Heart disease ☐Osteoporosis

☐ Asthma ☐ Cancer ☐Liver Disease ☐ Kidney Disease

☐Others: …….

☐Diabetes 1st degree relatives *mother father siblings children*

2nd degree relatives *grandparents uncle(mother) uncle(father) grandchildren*

☐ GDM 1st degree relatives *mother sister daughter*

2nd degree relatives *grandmother aunt(mother) aunt(father) grandchildren*

☐ Obesity 1st degree relatives *mother father siblings children*

2nd degree relatives *grandparents uncle(mother) uncle(father) grandchildren*

Medical history: Subject

☐HTN ☐Hyperlipidemia ☐Polycystic ovarian syndrome ☐Osteoporosis

☐ Asthma ☐Anemia ☐ GDM ☐ IGT ☐ Obesity

☐Others: …….

Age of menarche: …….

Menstrual cycle ☐ Regular ☐ Irregular

Age at first pregnancy: …….

LMP (last menstrual period): …… EDD (expected date of delivery) by date: ……

Risk Factors:

-The head circumference of last baby……

-Usage of insulin or its alternatives ☐Yes ☐ NO

-Parity (how many times she got pregnant): …… Number of children……

- Previous caesarean section ☐Yes ☐ No, how many…….

-Miscarriages if occurs?☐ Yes ☐ No If yes, how many times?.....Reasons……

-Preeclampsia ☐ Yes ☐ No -Gestational HTN ☐ Yes ☐ No

If any occurred in the past: ☐ Poly urea ☐ Glycosuria ☐ Protein urea

-Pregnancy symptoms:

☐Nausea ☐ vomiting ☐ morning sickness ☐headache ☐ mood swings

☐abdominal bloating ☐ frequent urination ☐constipation ☐ tender swallow breast

-Pregnancy complications: ……..

List of medications:

Please a check on all medications used by the subject

Anti-Hyperlipidemics	[ ] Mexiletine – Novo – Mexiletine
[ ] Atorvastatin – Lipitor, Torvast	[ ] Procainamide – Procan
	[ ] Propafenone – Rhythrnol, Nu, Apo, Gen, PMS

[ ] Cerivastatin – Lipobaby [ ] Fluvastatin – Lescol, Lescol XL [ ] Lovastatin – Mevacor, Altocor, Altoprev [ ] Mevastatin [ ] Pitavastatin – Livalo, Pitava [ ] Pravastatin – Pravachol, Selektine, Lipostat [ ] Rosuvastatin – Crestor [ ] Simvastatin – Zocor, Lipex [ ] Simvastatin + Ezetimibe – Vytorin [ ] Atorvastatin + Amlodipine – Caduet [ ] Simvastatin + Niacin – Simcor [ ] Cholestyramine [ ] Gemfibrozil [ ] Colestipol [ ] Benzafibrate, fenofibrate ***Cardiovascular drugs***	[ ] Digoxin [ ] Clonidine [ ] Methyldopa [ ] Diazoxide [ ] Hydralazine [ ] Isosarbide dinitrate [ ] Nitroglycerin [ ] Prazosin, Terazosin, Dozazosin [ ] Atenolol, Acebutolol, Bisoprolol, Labetalol, Metaoprolol, Nadolol, Oxprenolol, Pndolol, Propranolol, Sotalol, Timolol [ ] Amlodipine, Felodipine, Nifedipine [ ] Diltiazem, Verapamil [ ] Captopril, Benazepril, Enalapril, Cilazapril, Perindopril, Quinapril, Ramipril, Lisinopril [ ] Candesartan, Irbesartan, Losartan, Telmisartan, Valsartan, [ ] Spironolactone [ ] Hydrochlorothiazide [ ] Furosemide [ ] Pentoxyphylline ***Other (Please specify)*** 1. _____ 2. _____ 3. _____

[ ] Aspirin [ ] Warfarine [ ] Heparin [ ] Clopidogrel [ ] Amiodarone – Amiodarone, Cordarone [ ] Disopyramide – Rythmodan [ ] Flecainide acetate – Apo – Flecainide	

Second visit questionnaires		
Research code:	Date of filling of the form:	Date of filling of the form:
Name: Tel or Mobile No:	Place of birth: Week of gestation……	Date of Birth (age):
Anthropometric Measurements ❖ Ht.....cm Current Wt.....kg Prepregnancy Wt..... kg Current BMI……. kg/ m2 Before pregnancy BMI……. kg/ m2 ❖ **Blood Pressure**:.....		

Clinical Measurements
o Pregnancy symptoms: ☐Nausea ☐ vomiting ☐ morning sickness ☐headache ☐ mood swings ☐abdominal bloating ☐ frequent urination ☐constipation ☐ tender swallow breast
o Any complains or diagnosis…… ☐Gestational HTN ☐ Anemia ☐Viral or Bacterial illnesses ☐Preeclampsia ☐Respiratory ☐ Gastrointestinal ☐ bacterial vaginosis

Food Frequency Questionnaire														
Dietary intake of vitamin D and calcium Questionnaire														
Breakfast cereals and bread														
Daily					**Weekly**									**Amount**
○Cornflakes	1	2	3		1	2	3	4	5	6	M	R	No	……. Cup/ week
○Cornflakes with sugar and Coco Pops	1	2	3		1	2	3	4	5	6	M	R	No	…… Cup/ week
○ Weciabix, Shredded wheat	1	2	3		1	2	3	4	5	6	M	R	No	……. Cup/ week
○ Bran flakes, Wheat flakes or Sultana	1	2	3		1	2	3	4	5	6	M	R	No	……. Cup/ week
○ Brown Bread	1	2	3		1	2	3	4	5	6	M	R	No	……..toast/ week or ……..1/4 loaf
○White Bread	1	2	3		1	2	3	4	5	6	M	R	No	……..toast/ week or ……..1/4 loaf

○White Bread(Samoli,burger)	1	2	3		1	2	3	4	5	6	M	R	No	……..toast/ week or ……..1/4 loaf
○Shaboorah (bran or white)	1	2	3		1	2	3	4	5	6	M	R	No	……..pieces/week
*M (1-3 times per month), R(rarely), NO (don't take it at all)														
Meat and Fish														
Daily					**Weekly**									**Amount**
White fish (hamoor), fish fingers	1	2	3		1	2	3	4	5	6	M	R	No	…… gm Fried / Grilled
Oysters, shrimp	1	2	3		1	2	3	4	5	6	M	R	No	…… gm Fried / Grilled
Canned fish (canned salmon, sardines,)	1	2	3		1	2	3	4	5	6	M	R	No	…… piece With oil / water
Tuna fresh or canned	1	2	3		1	2	3	4	5	6	M	R	No	Can/large /small With oil / water
Red meat (……)	1	2	3		1	2	3	4	5	6	M	R	No	….gm/week With fat Little fat Without fat
Chicken Type ……( )	1	2	3		1	2	3	4	5	6	M	R	No	….gm/week Skin/with out skin
○Liver, brain and kidneys	1	2	3		1	2	3	4	5	6	M	R	No	….gm/week With fat Little fat Without fat
Egg														
How many eggs do you eat?	1	2	3		1	2	3	4	5	6	M	R	No	……Egg/ week

What kind of eggs do you eat? ☐ yolk ☐ White ☐ Both														
Fats														
What kind of fats used in your food? ☐ Ghee ☐ Margarine ☐ Butte ☐ Vegetables oil														
Daily					**Weekly**									**Amount**
○How many times do you use veg oil? Type….	1	2	3		1	2	3	4	5	6	M	R	No	……..Spoon / week
○How many times do you use olive oil?	1	2	3		1	2	3	4	5	6	M	R	No	……..Spoon / week
○How many times do you use butter or ghee? Type….	1	2	3		1	2	3	4	5	6	M	R	No	……..Spoon / week
○How many times do you eat Tahini? Type….	1	2	3		1	2	3	4	5	6	M	R	No	……..Spoon / week
○How many times do you usually have almonds or peanuts? Type……	1	2	3		1	2	3	4	5	6	M	R	No	……..Cup / week
*Table spoon (15 ml), cup (240 ml)														

Dairy And its Products														
<u>Do you usually drink milk?</u> ☐Yes ☐ No														
<u>If milk is liquid of which type?</u> ☐ Cow ☐ Camel ☐ Goat ☐ Sheep ☐ Other														
How many times do you drink fresh milk?														
Daily					**Weekly**									**Amount**
Full fat	1	2	3		1	2	3	4	5	6	M	R	No	……… Cup/ week

Low fat	1	2	3		1	2	3	4	5	6	M	R	No	.....Cup/ week
Skim fat	1	2	3		1	2	3	4	5	6	M	R	No	.....Cup / week
○ How many times do you drink dried milk?														
Daily					**Weekly**									**Amount**
Full fat	1	2	3		1	2	3	4	5	6	M	R	No	..... Cup/ week
Low fat	1	2	3		1	2	3	4	5	6	M	R	No	.....Cup/ week
Skim fat	1	2	3		1	2	3	4	5	6	M	R	No	.....Cup / week
○ How many time do you drink canned milk?														
Daily					**Weekly**									**Amount**
Full fat	1	2	3		1	2	3	4	5	6	M	R	No	..... Cup/ week
Low fat	1	2	3		1	2	3	4	5	6	M	R	No	.....Cup/ week
Full fat	1	2	3		1	2	3	4	5	6	M	R	No	.....Cup / week
Concentrated milk	1	2	3		1	2	3	4	5	6	M	R	No	..... Cup/ week
Chocolate	1	2	3		1	2	3	4	5	6	M	R	No	.....Cup/ week
With Strawberry	1	2	3		1	2	3	4	5	6	M	R	No	.....Cup / week
With Banana	1	2	3		1	2	3	4	5	6	M	R	No	..... Cup/ week
other	1	2	3		1	2	3	4	5	6	M	R	No	.....Cup/ week
○How many times do you drink Laban?														
Daily					**Weekly**									**Amount**
Full fat	1	2	3		1	2	3	4	5	6	M	R	No	..... Cup/ week
Low fat	1	2	3		1	2	3	4	5	6	M	R	No	..... Cup/ week
Skim fat	1	2	3		1	2	3	4	5	6	M	R	No	..... Cup/ week

How many times do you have yoghurt?

Daily				Weekly									Amount
Full fat	1	2	3	1	2	3	4	5	6	M	R	No	……. Cup/ week
Low fat	1	2	3	1	2	3	4	5	6	M	R	No	……. Cup/ week
Skim fat	1	2	3	1	2	3	4	5	6	M	R	No	……. Cup/ week

○How many times do you have these products?

Daily				Weekly									Amount
Triangle Cheese	1	2	3	1	2	3	4	5	6	M	R	No	……Piece/ week
Kerry Cheese	1	2	3	1	2	3	4	5	6	M	R	No	……Piece/ week
White Cheese	1	2	3	1	2	3	4	5	6	M	R	No	……Spoon / week
Yellow cheese	1	2	3	1	2	3	4	5	6	M	R	No	……Spoon / week
Fatty cheese	1	2	3	1	2	3	4	5	6	M	R	No	……Spoon / week
Geshtah	1	2	3	1	2	3	4	5	6	M	R	No	……Spoon / week
Labnah	1	2	3	1	2	3	4	5	6	M	R	No	……Spoon / week
Cream	1	2	3	1	2	3	4	5	6	M	R	No	……Spoon / week
Mozzarell a	1	2	3	1	2	3	4	5	6	M	R	No	……..gm/ week
Tofu	1	2	3	1	2	3	4	5	6	M	R	No	……Piece / week

* Food spoon= 15 gm

Vegetables and Fruits

○How many times do you eat the following?

Daily				Weekly									Amount
Orange	1	2	3	1	2	3	4	5	6	M	R	No	……One/ week

Fig	1	2	3		1	2	3	4	5	6	M	R	No	…….One/ week
Dried fruits (raisins,peache)	1	2	3		1	2	3	4	5	6	M	R	No	……..Cup / week
(fresh or cooked) kale	1	2	3		1	2	3	4	5	6	M	R	No	……..Cup / week
(fresh or cooked) celery	1	2	3		1	2	3	4	5	6	M	R	No	……..Cup / week
Spinach (fresh or cooked)	1	2	3		1	2	3	4	5	6	M	R	No	……Cup / week
(fresh or cooked) broccoli	1	2	3		1	2	3	4	5	6	M	R	No	……Cup / week
Mashroom (fresh or sundried)	1	2	3		1	2	3	4	5	6	M	R	No	……..Cup / week
Potatoes (fried or cooked)	1	2	3		1	2	3	4	5	6	M	R	No	…….One/ week
Homos,lentis	1	2	3		1	2	3	4	5	6	M	R	No	……Cup / week
Fool (black beans)	1	2	3		1	2	3	4	5	6	M	R	No	……Cup / week
*Cup = 240 m														

Cultural Food and others

	Weekly									**Amount**
How many times do you have Greesh per week?	1	2	3	4	5	6	M	R	No	……..Cup/ week
How many times do you have Saleeq per week?	1	2	3	4	5	6	M	R	No	……..Cup/ week
How many times do you have Harees per week?	1	2	3	4	5	6	M	R	No	……..Cup/ week

How many times do you have Marase or msabeb per week?	1	2	3	4	5	6	M	R	No	……..Cup/ week
How many times do you have Pasta with cheese or milk (lasagna, fettuccine, bashamel) per week?	1	2	3	4	5	6	M	R	No	……..Cup/ week
How many times do you have pizza per week?	1	2	3	4	5	6	M	R	No	……..pieces/week

Sweets

Daily				**Weekly**									**Amount**
How many times do you have Ice cream?	1	2	3	1	2	3	4	5	6	M	R	No	……Piece/ week Type……
How many times do you have carbonated beverages?	1	2	3	1	2	3	4	5	6	M	R	No	……Piece/ week Type……
How many times do you have chocolate?	1	2	3	1	2	3	4	5	6	M	R	No	……Piece/ week Type……
How many times do you have muffins or cake or donuts?	1	2	3	1	2	3	4	5	6	M	R	No	……Piece/ week Type……
How many times do you have biscuits?	1	2	3	1	2	3	4	5	6	M	R	No	…….Piece/ week Type……
How many times do you have cream caramel or custard?	1	2	3	1	2	3	4	5	6	M	R	No	……Piece/ week Type……

Tea and Coffee

Daily				Weekly									Amount
How many times do you drink red tea?	1	2	3	1	2	3	4	5	6	M	R	No	.....Cup/ week Type (.....)
How many times do you drink green tea?	1	2	3	1	2	3	4	5	6	M	R	No	.....Cup/ week Type (.....)

Current Season	☐Summer ☐Winter
What is the extent of your exposure to the sun?	☐ No exposure Daily Minutes (.....) ☐Weekly Minutes (.....)
What is the time of exposure to the sun?	☐ at sunrise (☐at noon) ☐ before sunset
What is the nature of your work?	☐Outside/ directly under the sun ☐Inside (in covered rooms/ paces)

Water			
	Daily		Amount
How much water do you drink?	1-3 cups	3-6 cups 6-12 cups	More than 12 cups
Kind?	Bottled Water	water from Tanks Tape water	

What are the parts of the body most exposed to the sun?	☐Face ☐hands ☐Face and hands ☐Face, hands and feet
What is the extent of clothing cover to the body during exposure to the sun?	☐Body except face ☐Body except face and hands ☐ Body except face, hands and feet ☐The whole body ☐Body except hands and feet
Do you use sun protection creams?	☐ Yes (type……) ☐ No

Physical activity (International Physical Activity Questionnaire) (IPAQ)
READ: I am going to ask you about the time you spent being physically active in the last 7 days. Please answer each question even if you do not consider yourself to be an active person. Think about the activities you do at work, as part of your house and yard work, to get from place to place, and in your spare time for recreation, exercise or sport. **READ: Now, think about all the *vigorous* activities which take *hard physical effort* that you did in the last 7 days. Vigorous activities make you breathe much harder than normal and may include heavy lifting, digging, aerobics, or fast bicycling. Think only about those physical activities that you did for at least 10 minutes at a time.** 1. During the last 7 days, on how many days did you do vigorous physical activities? _____ Days per week [VDAY; Range 0-7, 8,9] 8. Don't Know/Not Sure 9. Refused
2. How much time did you usually spend doing vigorous physical activities on one of those days? __ __ Hours per day [VDHRS; Range: 0-16] __ __ Minutes per day [VDMIN; Range: 0-960, 998, 999] 998. Don't Know/Not Sure 999. Refused
➔Interviewer probe: An average time for one of the days on which you do vigorous activity is being sought. If the respondent can't answer because the pattern of time spent varies widely from day to day, ask: "How much time in total would you spend over the last 7 days doing vigorous physical activities?" __ __ Hours per week [VWHRS; Range: 0-112] __ __ Minutes per week [VWMIN; Range: 0-6720, 9998, 9999] 9998. Don't Know/Not Sure 9999. Refused
READ: Now think about activities which take *moderate physical effort* that you did in the

last 7 days. Moderate physical activities make you breathe somewhat harder than normal and may include carrying light loads, bicycling at a regular pace, or doubles tennis. Do not include walking. Again, think about only those physical activities that you did for at least 10 minutes at a time.

3. During the last 7 days, on how many days did you do moderate physical activities?

____ Days per week [MDAY; Range: 0-7, 8, 9]
8. Don't Know/Not Sure
9. Refused

4. How much time did you usually spend doing moderate physical activities on one of those days?

__ __ Hours per day [MDHRS; Range: 0-16]
__ __ Minutes per day [MDMIN; Range: 0-960, 998, 999]
998. Don't Know/Not Sure
999. Refused

➔**[Interviewer probe: An average time for one of the days on which you do moderate activity is being sought. If the respondent can't answer because the pattern of time spent varies widely from day to day, or includes time spent in multiple jobs, ask: "What is the total amount of time you spent over the last 7 days doing moderate physical activities?"**

__ __ Hours per week [MWHRS; Range: 0-112]
__ __ Minutes per week [MWMIN; Range: 0-6720, 9998, 9999]
9998. Don't Know/Not Sure
9999. Refused

READ: Now think about the time you spent walking in the last 7 days. This includes at work and at home, walking to travel from place to place, and any other walking that you might do solely for recreation, sport, exercise, or leisure.

5. During the last 7 days, on how many days did you walk for at least 10 minutes at a time?

____ Days per week [WDAY; Range: 0-7, 8, 9] 8. Don't Know/Not Sure 9. Refused
6. How much time did you usually spend walking on one of those days? __ __ Hours per day [WDHRS; Range: 0-16] __ __ Minutes per day [WDMIN; Range: 0-960, 998, 999] 998. Don't Know/Not Sure 999. Refused
➔**[Interviewer probe: An average time for one of the days on which you walk is being sought. If the respondent can't answer because the pattern of time spent varies widely from day to day, ask: "What is the total amount of time you spent walking over the last 7 days?"** __ __ Hours per week [WWHRS; Range: 0-112] __ __ Minutes per week [WWMIN; Range: 0-6720, 9998, 9999] 9998. Don't Know/Not Sure 9999. Refused

READ: Now think about the time you spent sitting on week days during the last 7 days. Include time spent at work, at home, while doing course work, and during leisure time. This may include time spent sitting at a desk, visiting friends, reading or sitting or lying down to watch television.

7. During the last 7 days, how much time did you usually spend *sitting* on a week day?

__ __ Hours per weekday [SDHRS; 0-16]
__ __ Minutes per weekday [SDMIN; Range: 0-960, 998, 999]
998. Don't Know/Not Sure
999. Refused

➔**[Interviewer probe]: An average time per day spent sitting is being sought. If the respondent can't answer because the pattern of time spent varies widely from day to day, ask: "What is the total amount of time you spent *sitting* last Wednesday?"**

__ __ Hours on Wednesday [SWHRS; Range 0-16]
__ __ Minutes on Wednesday [SWMIN; Range: 0-960, 998, 999]
998. Don't Know/Not Sure
999. Refused

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